

EPIDEMIOLOGY OF *CRYPTOSPORIDIUM* SPP. IN CALVES AND DIARRHEA IN
LIVESTOCK AND HUMANS IN ETHIOPIA

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by

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EPIDEMIOLOGY OF *CRYPTOSPORIDIUM* SPP. IN CALVES AND DIARRHEA IN LIVESTOCK AND HUMANS IN ETHIOPIA

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The Apicomplexa genus *Cryptosporidium* is comprised of over 20 species to date. While *Cryptosporidium parvum* is host adapted to cattle it is the most frequently reported zoonosis in people. Cryptosporidiosis associated with *C. parvum* infection is characterized by osmotic malabsorptive diarrhea, which can be fatal in immune-compromised individuals. Diarrhea is the second leading cause of disease burden worldwide across all ages, and is the second leading cause of death worldwide in children under 5. Therefore, the objectives of this research were to:

- 1) Describe the probability of *Cryptosporidium parvum* fecal oocyst shedding at different magnitudes of exposure to *C. parvum*, the pattern of fecal shedding over time, and factors affecting fecal shedding in dairy calves;
- 2) Characterize the dose-response relationship of *Cryptosporidium parvum* in experimentally challenged dairy calves, and determine the median infective dose based on this relationship;
- 3) Describe common animal husbandry and manure management activities and the associated division of animal husbandry labor by age and sex, and determine the risk factors associated with reported history of diarrhea among Ethiopian agro-pastoralists in the Amhara Region.

It was determined that regardless of dose magnitude, all calves exhibited the same pattern of fecal shedding over time. There was a positive relationship between log-dose and diarrhea, and the ID₅₀ for fecal oocyst shedding with diarrhea was 16.6 oocysts. This indicates that the best means of controlling cryptosporidiosis is at the calf level, through the provision of clean dry housing and adequate nutrition. When studying diarrhea among agro-pastoralists in Ethiopia, history of diarrhea in people was not associated with history of diarrhea in livestock or type of animal husbandry system, but was associated

with household setting. There were also differences in division of animal husbandry labor, with women bearing the majority of the responsibility for manure management. This emphasizes the differences in potential disease exposure based on age and sex, the role of access to improved water sources in the prevention of diarrhea, and suggests that living in close proximity with livestock had no effect or may have a protective effect in some individuals with respect to diarrhea.

BIOGRAPHICAL SKETCH

Jennifer Ann Zambriski was born on Merritt Island in Rockledge, FL and spent most of her early years fishing with her father and then bathing with the leftover bait mullet. By the age of 7, Jennifer's father had taught her to name all the bones in her body as well as the complete cardiac physiology of the lizard. It was also Jennifer's dad who first told her that there was such a thing as an "animal doctor". From that moment on, Jennifer knew exactly what she wanted to be when she grew up.

Having earned an International Baccalaureate diploma, Jennifer graduated from Atlantic High School in Delray Beach, FL in 1996. Seeking to experience snow and public transportation, Jennifer headed north to Boston for college, earning a B.S. in Biology in 2000 from Tufts University. After graduation, Jennifer accepted a position with McMaster-Carr Industrial Supply in Atlanta, GA, where she was employed for 2 years as an Operational Manager. During this time, Jennifer became a volunteer at Zoo Atlanta, assisting in research projects with Asian Small Clawed River Otters and Giant Pandas. In 2002, Jennifer realized her life-long dream and matriculated at Tufts University Cummings School of Veterinary Medicine where she was accepted into the International Veterinary Medicine Certificate Program. During her time at Tufts, she conducted several international research projects, including studies of Nipah virus in Malaysian fruit bats and tuberculosis in Asian elephants in Nepal. Her studies also took her to Nicaragua to study brucellosis in cattle and to New Zealand to study small ruminant reproduction. As a veterinary student, Jennifer was a Geraldine R. Dodge Frontiers in Veterinary Medicine Fellow, as well as a Conservation Medicine Fellow with the Wildlife Conservation Society.

In 2006, upon earning a Doctor of Veterinary Medicine degree and Certificate in International Veterinary Medicine, Jennifer traveled to Ithaca to participate in the Summer Dairy Institute at Cornell University, where she met Dr. Daryl Nydam. Shortly after completing SDI, Jennifer returned to Ithaca in order to conduct research with Daryl focused on the development of a bovine vaccine against *Cryptosporidium parvum*, a zoonotic diarrheal parasite of dairy calves. She was hooked!

Jennifer left her post at Cornell in 2007 in order to accept a position with the United Nations Food and Agriculture Organization. As a Field Programme Facilitator with UN-FAO's Avian Influenza Control Programme, Jennifer lived and worked in Indonesia for a year, spending most of her time on Borneo conducting field research aimed at developing an Avian Influenza vaccination protocol in domestic ducks, but she missed her days in Ithaca with the calves and collaborating with Daryl, and knew she needed to return to Cornell and complete a Ph.D. in order to achieve the career she was envisioning.

Jennifer matriculated at Cornell in 2008 as a Ph.D. student in Animal Science. Her Ph.D. dissertation research has been focused on the epidemiology of *Cryptosporidium parvum* with application to control of cryptosporidiosis in livestock and humans in the Amhara Region of Ethiopia. During her tenure as a Ph.D. student, Jennifer was awarded the New York State Diversity Fellowship, a Fogarty International Clinical Research Scholarship through the National Institutes of Health, and an Integrated Graduate Education Research Traineeship in Food Systems and Poverty Reduction through the National Science Foundation. As a Fogarty Scholar Jennifer studied brucellosis in Peru, and as an NSF IGERT recipient, she conducted the final phase of her dissertation research on cryptosporidiosis in Ethiopia.

Upon completion of her Ph.D., Jennifer will join the faculty of Washington State University as a veterinary epidemiologist in the Allen School for Global Animal Health, where she plans to continue studying the human-animal health continuum in developing countries. Jennifer's research will focus specifically on the roles of livestock in the promotion of human health in western Kenya.

When she is not busy learning how to be a veterinary epidemiologist, Jennifer enjoys cooking, writing, and thinking up new names for OPI nail polish. But most of all, she enjoys the company and companionship of her dog, Sammy Bananas, and her cat, Roberto Handsome Paws.

For Grandpa Tat and Uncle Peanuts,
who loved me without condition and supported all of my academic endeavors. All I ever wanted
was to make them proud, and all they ever wanted was for me to know that they already were...

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To my vixens in Vestal and at VECCC, because of you my laugh lines are deeper and my sanity is intact. Beyond that, there are no words...at least not that I'm willing to commit to paper for fear of self-incrimination. You know who you are...

And of course, I wouldn't have made it this far if someone hadn't taken a chance on me in the very beginning. The mentorship and friendship of Dr. Gretchen Kaufman was essential to every part of this journey. I never believed that this life and career was possible or that I could find my way to it, and then GK shined a light on the path.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Introduction

The Apicomplexa-protozoan parasite genus *Cryptosporidium* is comprised of over 20 species, to date, with various host-adaptations, infecting 155 species of mammals (Fayer, 2004). Transmission of *Cryptosporidium* is by the fecal-oral route via contaminated water, food, or fomites; or by direct ingestion of infected feces. Cryptosporidiosis is the clinical syndrome of fever, diarrhea, and large volumes of fluid loss from the gastrointestinal tract (Fayer, 2004). The severity of this diarrhea and its subsequent dehydration influences morbidity and mortality in people and animals (O'Handley et al., 1999; Fayer, 2004)). Arguably, the most important of the *Cryptosporidium* spp. are *C. hominis*, which is host-adapted to humans, and *C. parvum*, which infects many mammals and is an important zoonosis (Fayer, 2004; Feltus et al., 2006; Ng et al., 2008; Xiao and Feng, 2008; Xiao, 2010).

There is not a consistently effective treatment for cryptosporidiosis in animals or in people. While Nitazoxanide has shown efficacy in reducing fecal oocyst shedding and improved clinical signs in experimentally infected dairy calves, there are no animal-labeled formulations currently available (Ollivet et al., 2009). Infection in healthy individuals is self-limiting, but individuals who are immunocompromised often develop intractable diarrhea, and die of severe dehydration (Xiao, 2008). The prevalence of cryptosporidiosis varies with immune status and differs between developed and developing nations. In immune-competent individuals living in developed countries the prevalence of cryptosporidiosis is 0.3 – 4.3% and in immune-compromised individuals it is 3.8 – 6.6% (Fayer, 2004; Hunter, 2002). This differs dramatically when compared to developing nations, where the prevalence in immune-competent individuals is 1.3 – 31.5% and in immune-compromised individuals it is 25 – 76% (Fayer, 2004; O'Connor, 2011). The differences in cryptosporidiosis prevalence between developed and developing countries illustrates that fact that poverty plays an important role in disease burden. Globally, 18% of the population subsists on less than \$1/day, and this population is reported to “bear a disproportionately high share of the burden of (zoonotic) disease”, which includes infection with *Cryptosporidium* spp. as well as other zoonotic pathogens (WHO, 2006).

It is possible to reduce the global burden of disease through improved livestock health, not only through the direct effects of reduced zoonotic disease exposure, but also through the indirect effects of improved livelihoods. In 2003, The International Livestock Research Institute published a report in which they delineated “pathways out of poverty” and specifically emphasized the important role of livestock for achieving this end. In order to promote human health and well-being, livestock health and well-being must also be sustained. Healthy livestock populations benefit the poor directly through reduced transmission of zoonotic disease, and improved nutrition through the provision of animal source protein. They also serve to buffer households against shocks, increase household income, and improve access to markets. When these elements are considered within a fluid system, it becomes evident that livestock aid the poor in the recovery of human capital and in turn provide a means of poverty alleviation.

The Biology of *Cryptosporidium*

Cryptosporidium spp. are obligate intracellular parasites that undergo sexual and asexual reproduction (Anderson, 1998; Fayer, 2004). The parasite life cycle is completed within the host animal via the formation of oocysts; small (4-6 μm) spherical structures which each contain 4 sporozoites. Oocysts are excreted from the gastrointestinal tract of infected hosts (people, calves, or other mammals) and are readily infective. Once ingested, sporozoites are released from the oocyst and invade the microvillus brush border of the small intestinal (and occasionally, colonic) enterocytes, which causes destruction of the adjacent enterocyte and villus atrophy, villus fusion, and intestinal crypt inflammation. This intestinal pathology creates a malabsorptive, maldigestive, and osmotic diarrhea that may or may not be secretory in nature (Saini et al., 2000; Elitok et al., 2005). It is believed that two types of oocysts are produced: those with a thick wall and those with a thin wall. The thin-walled oocysts, being more fragile, are capable of releasing sporozoites within the lumen of the host’s intestine before being excreted in the feces. This process, known as “auto-infection”, results in re-infection of the epithelial cells and initiates a new cycle of parasite development. Auto-infection is suspected to be the primary reason why immunocompromised individuals develop persistent intractable infections.

Notably, *Cryptosporidium* oocysts are highly resistant to extremes in temperature and to many commonly used disinfecting agents, therefore allowing them to persist in the environment for extensive periods of time, under a variety of environmental conditions, and remain infective (Robertson et al., 1992). Mice dosed with *C. parvum* oocysts stored at -10°C for 8 to 168 hours all became infected (Fayer and Nerad, 1996). In addition, *C. parvum* oocysts stored in water at 25°C for 12 weeks also remained infective (Fayer et al., 1996). Likewise, the infectivity of *C. parvum* oocysts was not reduced when oocysts were exposed for 33 minutes to 6% sodium hypochlorite, 70% ethanol, 37% methanol, 70% isopropanol, 6% hydrogen peroxide, or either of two ammonia-based laboratory disinfectants (Weir et al., 2002).

Molecular identification of *Cryptosporidium*

Since *Cryptosporidium* is ubiquitous in the environment, present in soil and water, and is characterized by numerous species infecting many different mammals; it is hard to know how infections originate and are transmitted. Beginning with Tyzzer's first report in 1907, and continuing for many decades, descriptions of *Cryptosporidium* spp. were limited to light microscopy (Tyzzer, 1907). With the advent of advanced molecular techniques, it has become possible to detect and differentiate *Cryptosporidium* spp. at the species/genotype and subtype levels. This is important for determining if *Cryptosporidium* infections are zoonotic or anthroponotic, if mixed infections are present, and if there is immunity and cross protection between species. Molecular knowledge also allows for the identification of species specific traits. For example, parasite-level factors in transmission dynamics and the resultant spectrum of clinical illness in the host are important to elucidate at the molecular level as one subtype may be more virulent than another.

Currently, *Cryptosporidium* spp. isolated from people, water, and animals are most commonly differentiated using the small subunit (SSU) rRNA gene (Xiao et al., 1999; Xiao et al., 2001). The *Cryptosporidium* oocyst wall protein (COWP) gene is also used, though rarely, and sometimes in conjunction with the SSU rRNA gene. The SSU rRNA gene tends to be preferred as it has semi-

conserved and hyper-variable regions which aid in the design of genus-specific primers. COWP based tools tend to have narrow specificity and therefore are less useful when genotyping *Cryptosporidium* spp. in animals (Xiao, 2010). Subtyping tools are useful in differentiating the transmission dynamics of *C. hominis* and *C. parvum*. The DNA sequence encoding the 60 kDa glycoprotein (gp60) gene is preferred as it has variations in the number of trinucleotide repeats, which is important for differentiating one subtype family from another (Xiao, 2010).

When applying these tools it is important to consider the specimen and the order in which the tools are applied. Regardless of which Polymerase Chain Reaction (PCR) tool is selected, there is a bias toward detecting the dominant genotype in the specimen, as the tools are broadly specific. This effect is compounded by the nature of PCR amplification, and the need for a large amount of PCR product to be visible on an agarose gel. Conversely, subtyping tools for *C. hominis* and *C. parvum* are narrowly specific, and may fail to detect concurrent infections. Cama *et al.* (2006) detected *C. canis* and *C. felis* in specimens for HIV+ Peruvian patients when using SSU rRNA-based PCR- Restriction Fragment Length Polymorphism (RFLP) genotyping techniques. When the same specimens were analyzed using COWP- and dihydrofolate reductase-based PCR-RFLP tools, they failed to detect *C. canis* and *C. felis* and detected *C. hominis*, *C. parvum*, or *C. meleagridis* instead (Cama *et al.*, 2006).

***Cryptosporidium* in dairy cattle**

In cattle, there are three species of *Cryptosporidium* of particular importance: *C. bovis*, *C. andersoni*, and *C. parvum*. Each of these different species is prevalent in cattle at different ages and has varying degrees of zoonotic potential. Among these three species, *C. bovis* and *C. andersoni* are prevalent in weaned and adult cattle, respectively, and are not important zoonoses (Fayer *et al.*, 2007). *C. parvum*, however, is an important zoonosis, is the most pathogenic species in cattle, and is prevalent in calves before weaning (Lindsay *et al.*, 2000; Santin *et al.*, 2004; Robinson *et al.*, 2006; Trotz-Williams *et al.*, 2006; Fayer *et al.*, 2007).

It has been estimated that approximately 60 to 90% of dairy herds in North America have at least one calf infected with *C. parvum* (Garber et al., 1994; Trotz-Williams et al., 2008). Since the oocysts are readily infective and can survive for several weeks to months in the environment, infection most likely occurs when calves are born into contaminated maternity pens or are housed within contaminated calf hutches (Anderson, 1998). *Cryptosporidium* is one of the major etiologic agents of neonatal diarrhea complex and a main cause of calf-hood morbidity and mortality (de la Fuente et al., 1999; Naciri et al., 1999). Health of infected calves ranges from being clinically normal with fecal shedding, to having severe diarrhea, anorexia, and secondary dehydration (Moore and Zeman, 1991; Jarvie et al., 2005). Infection and disease most commonly occur in calves between 1 and 4 weeks of age, and usually lasts for approximately 2 weeks, however, low levels of oocyst shedding in the feces of older animals has been reported (Xiao and Herd, 1994; de Graaf et al., 1999; O'Handley et al., 1999). In young animals, during the first few days of infection, large numbers of oocysts may be shed in the feces. It has been determined that a naturally infected 6-day-old calf that sheds oocysts for 6 days, produces an estimated 1,028,786 oocysts per gram of feces (Nydham et al., 2001).

In neonatal dairy calves, *C. parvum* is one of the leading causes of cryptosporidiosis and contributes to substantial economic losses (Lefay et al., 2000; Trotz-Williams et al., 2007). On-farm management goals for the control of cryptosporidiosis primarily focus on cleanliness of maternity pens, calf housing and feeding equipment, separation of dam and calf at birth, as well as early detection of anorexia, diarrhea, and dehydration in neonatal calves (Harp and Goff, 1998; Nydam and Peregrine, 2005). Treatment is supportive, focusing on prevention and correction of fluid and energy deficits and electrolyte disturbances associated with the diarrhea. There is neither an approved product nor an extra-label product available in the United States that has shown consistent efficacy toward reducing *C. parvum*-associated diarrhea or fecal shedding of oocysts in dairy calves (Harp and Goff, 1995; Nydam and Peregrine, 2005). There are also currently no commercially available vaccines for use in animals or people. Moore *et al.* (2003) conducted a study examining the efficacy of prophylactic decoquinate for the

treatment of calves experimentally infected with *C. parvum*, and found no significant difference in clinical signs or oocyst shedding associated with cryptosporidiosis (Moore et al., 2003). Nitazoxanide, on the other hand, was effective in reducing fecal oocyst shedding and improved clinical signs in experimentally infected dairy calves; however, there are currently no animal-labeled formulations available (Ollivett et al., 2009). Animal-labeled formulations of halofuginone are commercially available, however, while halofuginone does improve clinical signs by delaying the onset of diarrhea and reducing fecal oocyst shedding, it does not prevent diarrhea and fecal shedding entirely (Jarvie et al., 2005).

***Cryptosporidium* in people**

Transmission of *Cryptosporidium* is by the fecal-oral route. Cryptosporidiosis is the clinical syndrome of fever, diarrhea, and large volumes of fluid loss from the gastrointestinal tract (Fayer, 2004). The severity of this diarrhea and its subsequent dehydration influences morbidity and mortality in people and animals (O'Handley et al., 1999; Fayer, 2004). The most important of the *Cryptosporidium* spp., with respect to human infection, are *C. hominis*, which is host-adapted to humans, and *C. parvum*, which is the most frequently reported zoonotic species in people (Fayer, 2004; Feltus et al., 2006; Ng et al., 2008; Xiao and Feng, 2008; Xiao, 2010).

In order to better understand the natural history of *C. hominis* and *C. parvum*, their infectivity, and the host immune response, experimental studies in healthy adult volunteers have been conducted. For *C. parvum* infection, the number of oocysts ingested did not influence the onset or duration of clinical illness, but a significant positive relationship between the size of the inoculum and occurrence of enteric symptoms was reported (DuPont et al., 1995). Thus, the larger the exposure to infective oocysts, the worse the severity of clinical illness. The same also holds true for *C. hominis* infection in healthy human volunteers and a positive dose-response relationship has been described. Individuals who received lower doses of *C. hominis* were less likely to experience diarrheal illness (Chappell et al., 2006).

The median infective dose of *C. parvum* in people was determined to be 132 oocysts (DuPont et al., 1995). In people experimentally infected with *C. parvum*, 61% developed enteric symptoms, and 39% were considered to have clinical cryptosporidiosis (DuPont et al., 1995). Similarly, in people dosed with *C. hominis*, 61.9% developed enteric symptoms (Chappell et al., 2006). The median infective dose for *C. hominis* in people ranges between 10 and 83 oocysts, depending upon the definition of infection being applied (Chappell et al., 2006). A microbiologic definition of oocyst shedding without or without diarrhea is associated with an ID₅₀ of 83 oocysts, and clinical definition of diarrhea with or without detection of oocysts is associated with an ID₅₀ of 10 oocysts. It is interesting to note that the ID₅₀ for *C. hominis* in people is lower than the ID₅₀ for *C. parvum* in people. This is likely due to the fact that *C. hominis* is host adapted to people.

When the relatively low median infective dose in people is considered in conjunction with the large number of oocysts shed by calves, and the known positive dose-response relationship, it is reasonable to conclude that infected calves that are shedding the organism act as a potential source of infection for other susceptible animals and people. Likewise, infected people shedding *C. hominis* and *C. parvum*, act as potential zoonotic sources of infection.

Immunology and Vaccine Development

In order to develop chemotherapeutics and vaccines, the course of infection and host response must be thoroughly understood so that feasible physiological intervention points can be identified; allowing response to treatment and vaccine efficacy to be measured and evaluated. Given the recent development of a *C. parvum* vaccine (rCP15 vaccine) for use in pregnant cows, there is a strong rationale to describe the course of infection in healthy calves, as well as the host immune response. These data can be used to determine a median infectious dose for calves, and this dose can be used in experimental challenge trials to test vaccine efficacy.

While experimental challenge trials are important in elucidating the natural history of *C. parvum* infection, the knowledge gained in these trials is significantly enhanced when interpreted in conjunction with molecular data. Recent genotype studies have indicated that calves are the only major reservoir of *C. parvum* for human infection (Xiao and Feng, 2008). Human *C. parvum* infections are particularly prevalent and often fatal in neonates in developing countries and immunocompromised persons such as AIDs patients (Xiao and Feng, 2008). Numerous studies in mice have shown that a type 1 immune response with CD4⁺ T cell production of IFN- γ is essential for protection (Wyatt and MacDonald, 2004). Additionally, while studies in the cows are limited, it appears that a type 1 immune response also is protective (Wyatt et al., 2001).

Chemotherapeutics against cryptosporidiosis are limited and not wholly efficacious in either humans or calves, making development of an effective vaccine of paramount importance (Gargala, 2008). To date, there is no commercially available effective vaccine against *C. parvum*, although passive immunization utilizing different zoite surface (glyco) proteins has shown promise (Fayer et al., 1989; Harp and Goff, 1995; Perryman et al., 1999; Sagodira et al., 1999). The 15 kDa 123 amino acid antigen of *C. parvum* designated CP15 (formerly CP15/60) (GenBank Accession No. L34568) was identified by Jenkins and Fayer (Jenkins and Fayer, 1995). CP15 is expressed by the infective sporozoite and merozoite stages on the oocyst surface as well as other internal structures (Boulter-Bitzer et al., 2007). The gene encoding the CP15 antigen has been cloned and expressed as a recombinant protein (rCP15) and used in combination with aluminum hydroxide as a vaccine candidate to decrease transmission and disease severity in calves. Recently, it has been demonstrated that heifers vaccinated with rCP15 produced a significantly greater antibody response compared to controls and this response was strongly associated with the subsequent level of colostral antibody (Burton et al., 2010). Furthermore, calves fed rCP15-immune colostrum showed a dose-dependent absorption of antibody, also associated with colostral antibody levels (Burton et al., 2010).

Taken together, these data demonstrate that vaccination induces an immune response in heifers, specific antibodies are secreted into their colostrum, and calves can absorb these antibodies. In light of this information, further characterization of calf immune response in the presence and absence of immune colostrums is necessary in order to determine if the rCP15 vaccine will be a practical method for conferring reliable passive protection to calves against cryptosporidiosis and will modulate immunity to limit tissue destruction. While there are important differences in calf immune response to parasitic and viral infection, it is worth noting that Parreno *et al.* reported an altered immune response in rotavirus infected calves that received immune colostrum (Parreno et al., 2004).

The Relationship between *Cryptosporidium* and Water

Water is the major vehicle for transmission of cryptosporidiosis (Fayer, 2004). Previously, it was believed that the presence of *Cryptosporidium* oocysts in surface water was infrequent and limited to geographically isolated locations, however, this has been disproven (Rose et al., 1997). To the contrary, *Cryptosporidium* oocysts are present in both surface and ground water, with frequencies reported at 4-100% and 9.5-22%, respectively (Hancock et al., 1998). Between 1984 and 1999, approximately 49 drinking water related outbreaks were reported in North America, the United Kingdom, and Japan where detection and monitoring systems are in place (Fayer et al., 2000). The largest known outbreak for any water-borne pathogen ever recorded occurred in 1993 in Milwaukee, Wisconsin and was attributed to *Cryptosporidium*; although the species was not identified. Cryptosporidiosis was reported in approximately 403,000 people (Mac Kenzie et al., 1994). Over the course of several years, the outbreak was attributed to dairy farm runoff, drainage from an abattoir, and other sources. In 1997, Peng *et al* conducted molecular analysis on stored fecal samples from the outbreak and identified oocyst-derived DNA that was identical to the *Cryptosporidium* human genotype, thus indicating that the source of the outbreak was from human feces and not animal feces (Peng et al., 1997). This genotype is now recognized a separate species, *C. hominis* (Morgan-Ryan et al., 2002).

The Relationship between Livestock Health and Poverty in Resource-Poor Settings

When considering the relationship between human and animal health, disease transmission has traditionally been of greatest concern. Parasites, bacteria, fungi, and viruses that travel between people and animals are frequently implicated, and rightfully so, as they negatively impact people and animals alike. In an attempt to describe the relationship between human and animal health, scientists have focused on understanding how livestock cause disease with insufficient emphasis on how livestock promote well-being. Historically, the domestication of livestock was a means to stabilize the food supply, and in current development settings, the same holds true. However, the relationship between people and livestock has shifted and intensified; its complexity extending beyond the well circumscribed boundaries of food and disease. Livestock have emerged not just as a food source, but also as sources of organic matter and traction, as well as financial institutions or “walking savings accounts”. In these roles they maintain human capital and provide pathways out of poverty. In 2003, the International Livestock Research Institute published a report in which they delineated these “pathways out of poverty” and identified three main livelihood strategies, and the role of livestock in particular, for achieving this end (International Livestock Research Institute, 2003). The pathways focus on the ways in which livestock; 1) buffer households against shocks and allow them to bear risks associated with income generating strategies; 2) increase household income and promote asset accumulation through specialization and intensification of livestock production; and 3) improve access to markets and increase profitability of livestock (and associated livestock activities). While these pathways are logical, they are not attainable in the face of diminished human capital. Chronic illness and malnutrition directly impact the ability of the poor to complete daily tasks necessary for maintaining their livelihoods. Randolph *et al.* pose that through various mechanisms, livestock keeping positively impacts the physical well-being of the poor, and that the most direct mechanism is through improved nutrition (Randolph et al., 2007). This notion is supported by the fact that improved cognitive function in children is predicted by nutritional status and is

significantly associated with increased intake of dietary micronutrients and with dietary meat and milk supplementation (Neumann et al., 2007; Bangirana et al., 2009; Gewa et al., 2009).

Based on this information, it stands to reason that strong incentives exist to maintain livestock health and well-being in order to promote human health and well-being. Nevertheless, for all the advantages offered by livestock keeping, a measure of risk must be assumed. Livestock do pose health risks to the poor, primarily in the form of zoonoses and food-borne illnesses. In order for livestock keeping to truly provide “pathways out of poverty”, these risks must be mitigated. In 2006, WHO reported that the poor in fact “bear a disproportionately high share of the burden of (zoonotic) disease”, and this is likely attributed to the fact that the poor have more frequent and prolonged contact with livestock and their manure, have decreased access to healthcare, have poor personal hygiene, have reduced access to potable water, and often live in unsanitary conditions (WHO, 2006). These factors are multifaceted and pose increased opportunities for disease transmission. In developed countries, these risk factors have been identified, categorized, and mollified through public sector interventions. In development settings, it is unrealistic to expect public agencies to be able to successfully implement disease control measures which are not suitable to the theoretical or physical landscape of a developing nation. Political instability, lack of financial resources, and poor infrastructure reduce the capacity to deliver goods and services, veterinary or otherwise. Therefore, alternate zoonotic disease control strategies must be developed that complement existing animal husbandry systems, in order to increase the likelihood of adoption and sustainability.

Traditional knowledge and livestock-keeping

In order to create zoonotic disease control strategies that appropriately complement existing animal husbandry systems, efforts must be made to identify and incorporate traditional knowledge. Through the use of participatory measures, interventions can be developed in a way that complements existing systems and therefore fosters integration of the interventions into the existing systems. In

developed settings, where animals are confined and intensively managed, interventions emulate the management system and are easily implemented. In development settings, however, these measures are not transferable. For example, it would be unwise to ask a nomadic pastoralist to practice intensive confinement interventions, and it would be unrealistic to expect any degree of compliance. As it stands, nomadic pastoralism in and of itself could be viewed as an intervention. Herders move their livestock in order to provide them with access to food and water, and to minimize disease risks. Decisions pertaining to why livestock should be moved, when they should be moved, and where to move them are founded in traditional knowledge. A nomadic pastoralist, like any other livestock farmer, uses the information available to him in order to assess risk and assume risk. According to Turner *et al.*, specific practices within indigenous communities provides them with a greater diversity of cultural capital and in turn helps to maintain their flexibility and resilience; two traits which are necessary components of any successful community intervention (Turner et al., 2003). Therefore, when analyzing risk factors for disease in livestock and developing interventions, the generation of context-specific knowledge should be emphasized. This practice not only increases the likelihood of community adoption, but in certain indigenous community settings, may result in improved flexibility and resilience in adopted interventions. Through expanded understanding of the creative and pragmatic interaction between indigenous and scientific knowledge the likelihood of producing practical outcomes that will meet the urgent priorities of village communities is vastly improved (Kassam, 2009).

Cryptosporidiosis in Developing Nations

Diarrhea due to zoonotic disease in development settings is one of the greatest and most prevalent threats to human health and well-being. In a report on diarrheal illness in children from 2009, diarrhea is identified as the second leading cause of death in children under age 5 worldwide, with nearly 1 in 5 children affected (WHO, 2009). In rural farming areas, and in regions where *Cryptosporidium* spp. are endemic, children under the age of 5 have the greatest incidence of disease (Meinhardt et al., 1996; Majowicz et al., 2001). Zoonotic causes of diarrheal illness are often preventable through improved

sanitation and manure handling measures, however, in resource-poor settings, implementing these measures can be a challenge due to over-crowding and limited access to clean water. Zoonotic enteric pathogens such as *Salmonella* spp, *Campylobacter* spp, *Escherichia coli*, *Cryptosporidium* and *Giardia* are frequent causes of diarrheal disease (Acha and Szyfres, 2003). Contact with livestock and their manure is a commonly reported risk factor associated with transmission of these pathogens and with zoonotic diarrheal illness (Grados et al., 1988; Adah et al., 2002; Fayer, 2004; Rao Ajjampur et al., 2007; Coles et al., 2009; Febriani et al., 2009). Many zoonotic infections can be successfully treated with chemotherapeutic agents, provided that the medications are available within the community and are equitably distributed. However, given the general lack of access to medical care, the cost of medications, and the impact of prolonged illness on human capital; disease prevention is preferred.

In the case of cryptosporidiosis, there are no consistently effective treatments, however, individuals who are immunocompetent, are able to eliminate the parasite and recover (Xiao and Feng, 2008). Immunocompromised individuals, however, often develop intractable diarrhea, and die of severe dehydration. In developed countries, the prevalence of human infection is as low as 0.3%, and in developing countries it is as high as 31.5% (Fayer, 2004). This serves as evidence of the fact that in developed countries, where water treatment and sanitation are adequate, disease control measures can be effectively implemented; and illness in people can be prevented. However, in order for this to be achieved in developing nations, it is not only necessary to gain a better understanding of the parasite and its relationship with animal and human hosts, but to also better understand the geographic, cultural, and traditional influences on animal husbandry and manure handling that may result in increased risk of disease exposure and transmission between livestock and people.

In developed countries, virtually all cryptosporidiosis outbreaks, for which a cause could be determined, were attributed to deficiencies at water treatment plants (Fayer, 2004). However, in

developing countries, where water treatment is not common, the sources of outbreaks are quite different. This is evidenced by that fact that in Ethiopia the prevalence of cryptosporidiosis does not significantly differ among children drinking from protected and unprotected sources (Ayalew et al., 2008). Other studies conducted in the same village showed that on average 7 liters of water per capita per day were used for domestic purposes, and that this is not sufficient for adequate personal hygiene and food safety (Howard and Bartram, 2003; Scheelbeek, 2005). These findings suggest that children have been infected via other fecal-oral routes, and this can likely be attributed to contaminated food, lack of potable water, poor hygiene, or other risk factors that increase the frequency of contact with animal feces.

The seasonality of cryptosporidiosis is well described, with increased incidence occurring during the rainy season in tropical climates (Muchiri et al., 2009). Studies from Central America, South Africa, Kuwait, India, and Ethiopia all report peak incidence of cryptosporidiosis during the rainy season (Iqbal et al., 1999; Leach et al., 2000; Ayalew et al., 2008). Worldwide it is generally accepted that seasonal weather patterns affect the transmission cycles and seasonal peaks of other infectious diseases. These seasonal peaks likely will be affected by climate change. Jagai *et al* demonstrated that increases in temperature and precipitation predict increased incidence of cryptosporidiosis in certain geographic regions of the world. In Sub-Saharan Africa specifically, the combined effects of temperature and precipitation on vegetation predict disease (Jagai et al., 2009). Furthermore, in watersheds of Meru, Kenya, where water flows from intensively farmed land, through densely populated areas before reaching semi-arid pastoral areas, there is significant seasonality in surface water contamination with *Cryptosporidium* (Muchiri et al., 2009). In regions such as Bahir Dar, Ethiopia, which is situated on Lake Tana, the primary source of the Blue Nile, the effects of intensive farming, population density, and climate change are severely negatively impacting the regional ecosystem through erosion and severe siltation (Awulachew et al., 2008). It stands to reason that as climate change progresses and affects rainfall; the seasonal variation in cryptosporidiosis in these regions will also be impacted, though the degree and extent are currently unknown. As drought-flood cycles are altered, regional water-use will also

change in response to environmental conditions. During droughts, fecal contamination of communal water sources due to overcrowding of people and livestock is likely to occur. And in times of flooding, fecal contamination is a challenge as sewage overflow worsens, and flood waters infiltrate previously dry land, which will in turn maintain high moisture levels and contribute to environmental persistence of infective oocysts. Therefore, it is anticipated that these environmental variations will affect existing livestock husbandry systems and will be associated with increased risk of cryptosporidiosis. Identification of associated disease risk factors as they pertain to livestock husbandry systems is necessary in order to reduce disease risk. Knowledge gained through the identification of risk factors can be used to develop interventions that are capable of both accounting for the environmental variations, and complementing traditional husbandry methods.

Cryptosporidiosis and HIV in Ethiopia

Having approximately 74 million animals, Ethiopia's livestock population is the largest on the African continent, and is estimated to provide livelihoods for 65% of Ethiopians (Food and Agriculture Organization of the United Nations, 1999). Nutrition in children in the Amhara region is the poorest nationally with 56.6% of children under 5 being stunted (Macro International Inc., 2005). The prevalence of HIV in the region is 1.7% and the prevalence of *Cryptosporidium* in adult AIDS patients throughout Ethiopia is 25.9% (Macro International Inc., 2005; Adamu et al., 2006). It is well documented that in the absence of anti-retroviral therapy, adults in developing countries suffer from persistent diarrhea associated with cryptosporidiosis (Fisseha et al., 1998). However, relatively few reports exist in children. In one study conducted in nearby Uganda, among children hospitalized for diarrhea, 5.9% were reported to have infection with *Cryptosporidium parvum*, and this percentage increased to 73.6% in children with HIV (Tumwine et al., 2005).

Infection with *Cryptosporidium* predisposes children to persistent diarrhea, and is significantly associated with malnutrition; including unfavorable outcomes such as stunting, wasting, being under weight, and death (Tumwine et al., 2003). Cryptosporidiosis also contributes to impaired physical fitness

in late childhood (Tumwine et al., 2003; Eppig et al., 2010). In 2002, Amadi *et al.* reported that a 3 day course of Nitazoxanide significantly improved resolution of diarrhea, parasitological eradication, and mortality in HIV-seronegative, but not HIV-seropositive children (Amadi et al., 2002).

Contact with animal feces is a risk factor for cryptosporidiosis. While there are numerous reports documenting contact with animals as a risk factor for cryptosporidiosis, there are no reports that describe the nature and extent of this contact with respect to husbandry and manure management, nor the impact on HIV positive children (Fayer et al., 1997; Fayer, 2004). Since different types of husbandry and manure management systems require varying levels of contact with animals and their manure, it stands to reason that risk of cryptosporidiosis varies depending upon the type of livestock husbandry system. Through the identification of risk factors associated with existing husbandry systems and differences in disease incidence, it is possible to identify aspects of animal management associated with a lower risk of disease. Through simple manipulations of the existing husbandry system, interventions to mitigate disease exposure can be developed and tested.

Research Objectives

- 1) Describe the probability of *Cryptosporidium parvum* fecal oocyst shedding at different magnitudes of exposure to *C. parvum*, the pattern of fecal shedding over time, and factors affecting fecal shedding in dairy calves;
- 2) Characterize the dose-response relationship of *Cryptosporidium parvum* in experimentally challenged neonatal dairy calves, and determine the median infective dose based on this relationship;
- 3) Describe common animal husbandry and manure management activities and the associated division of animal husbandry labor by age and sex, and determine the risk factors associated with reported history of diarrhea among Ethiopian agro-pastoralists in the Amhara Region.

Rationale for thesis project

Epidemiology allows us to study interactions between the host, the pathogen, and the environment, to test those interactions, and then to measure them. Ultimately, the knowledge gained can be used to understand, anticipate, and alter the relationship between exposure and outcome to serve in the prevention of disease and in the promotion of wellbeing.

It is my belief that there is a human-animal health continuum, and, in the context of global health and development, this continuum plays an important role in the health and wellbeing of people and animals alike. In resource poor settings, livestock ownership is associated with zoonotic disease transmission, but owning livestock is also associated with helping to break the cycle of poverty. Therefore, by maintaining healthy livestock, we can improve the health of the individuals who depend upon them.

Ultimately, I hope that the information shared here will result in the ability to modify and enhance existing husbandry systems and permit the development of geographically and culturally appropriate interventions. Through the implementation of these interventions in developing countries with similar husbandry and manure management systems, it may be possible to reduce the zoonotic disease burden in vulnerable populations, minimize unfavorable health outcomes in HIV patients, and help the poor begin to recover human capital and begin and break the cycle of poverty.

Challenges such as those related to water management, manure systems, nutrition, markets, and political instability all serve to perpetuate the disease transmission cycle, and in turn, the poverty cycle. Nevertheless, the complex nature of the problem should not serve as a disincentive for the creative development of new solutions. When addressing issues of this nature, multidisciplinary and participatory methods serve to integrate and blend multiple dimensions thereby offering solutions that mirror the dynamics of the systems involved. These measures are essential in order to assure regional adoption and assimilation. In order for progress to be achieved, animal health must be established and maintained over generations. Solutions must not only be attainable, they must be replicable. In order for livestock to serve

as a pathway out of poverty, they must be reliably healthy and productive. While disease control is an important factor in livestock health and production, it is only one factor, and it can only be achieved in concert with many others.

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CHAPTER TWO

DESCRIPTION OF FECAL SHEDDING OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS IN EXPERIMENTALLY CHALLENGED DAIRY CALVES

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Abstract

The objective was to describe the probability of *Cryptosporidium parvum* fecal oocyst shedding at different magnitudes of exposure, the pattern of fecal shedding over time, and factors affecting fecal shedding in dairy calves. Within the first 24 hours of life, 36 calves were experimentally challenged with *C. parvum* oocysts at one of four possible magnitudes of oral exposure (1×10^3 , 1×10^4 , 1×10^5 , and 1×10^6 oocysts), and 7 control calves were sham dosed. Fecal shedding occurred in 33 (91.7%) experimentally challenged calves and in 0 control calves. There was a difference in the log-total number of oocysts counted/g of feces dry weight among the 4 exposure groups; calves with the lowest magnitude of exposure (1×10^3 oocysts) shed less than the other 3 groups. At higher magnitudes of exposure there was more variability in the range of fecal oocyst shedding. There was an inverse relationship between the log-total amount of oocysts counted/g of feces dry weight and the number of days to the onset of fecal shedding per calf, i.e., the more time that elapsed to the onset of fecal shedding, the fewer oocysts that were shed. The pattern of fecal shedding over time for all calves shedding oocysts was curvilinear; the number of oocysts increased with time, reached a peak, and declined. Therefore, the dynamics of oocyst shedding can be influenced in part by limiting exposure among calves and delaying the onset of fecal oocyst shedding.

Introduction

The Apicomplexa-protozoan parasite genus *Cryptosporidium* is comprised of over 20 species to date (Fayer, 2004). Transmission of *Cryptosporidium* is most often by the fecal-oral route via contaminated water, food, or fomites; or by direct ingestion of infected feces. In cattle, *C. bovis*, *C. andersoni*, and *C. parvum*, are species of particular importance. Among these 3 species, *C. bovis* and *C. andersoni* are prevalent in post-weaned and adult cattle, respectively, and are not important zoonoses (Fayer et al., 2007). In contrast, *C. parvum* is an important zoonosis, is the most pathogenic species in cattle, and is prevalent in pre-weaned calves (Lindsay et al., 2000; Santin et al., 2004; Robinson et al., 2006; Trotz-Williams et al., 2006; Fayer et al., 2007). *C. parvum* infects many mammals and is globally

recognized as a significant cause of diarrhea in people and cattle (Feltus et al., 2006; Xiao and Feng, 2008). *C. hominis*, which is host-adapted to humans, is also an important cause of diarrhea in people and was the cause of the largest water-borne disease outbreak ever recorded, impacting approximately 403,000 citizens of Milwaukee, Wisconsin in 1993 (MacKenzie et al., 1995; Morgan-Ryan et al., 2002; Chappell et al., 2006).

Cryptosporidiosis is the clinical syndrome of fever, diarrhea, and large volumes of fluid loss from the gastrointestinal tract (DuPont et al., 1995; Fayer, 2004). The severity of this diarrhea and its subsequent dehydration influences morbidity and mortality in people and animals (Anderson, 1998; O'Handley et al., 1999). Treatment of the illness poses a challenge as there is not a consistently effective chemotherapeutic agent for use in animals or in people (Harp and Goff, 1998; Amadi et al., 2002; Mead, 2002; Anderson and Curran, 2007; Graczyk et al., 2011). For example, nitazoxanide has shown efficacy in immune-competent but not immune-compromised children (Amadi et al., 2002). In experimentally infected dairy calves, treatment with nitazoxanide reduces the duration of fecal oocyst shedding and improves clinical signs, however, there are no animal-labeled formulations at this time (Ollivett et al., 2009). Animal-labeled formulations of halofuginone are available. While halofuginone has been shown to improve clinical signs by delaying the onset of diarrhea, and reducing fecal oocyst shedding, it does not prevent diarrhea and fecal shedding entirely (Jarvie et al., 2005).

In neonatal dairy calves, *C. parvum* is one of the leading causes of cryptosporidiosis and contributes to calf-hood diarrhea (Lefay et al., 2001; Trotz-Williams et al., 2007). Infection and disease most commonly occur in calves between 1 and 4 weeks of age, and usually lasts for approximately 2 weeks (Xiao and Herd, 1994; de Graaf et al., 1999; O'Handley et al., 1999). In young animals, during the first few days of natural infection, large numbers of oocysts may be shed in the feces. For example, a naturally infected 6-day-old calf that sheds oocysts for 6 days may produce in excess of 3×10^{10} oocysts (Nydam et al., 2001). Excreted oocysts are readily infective and can survive for several weeks to months

in the environment and likely cause infection when calves are born into contaminated maternity pens or placed in contaminated housing (Anderson, 1998). Therefore, the extent of calf exposure to infective oocysts may be associated with the degree of environmental contamination. There is currently little published information on this relationship in calves, nor is there literature describing the pattern of fecal oocyst shedding over time as it relates to the magnitude of exposure.

Currently, the relationship between the magnitude of oral exposure and fecal oocyst shedding is not well described in calves. In this study, we experimentally challenged neonatal dairy calves with *C. parvum* oocysts and report the probability of fecal oocyst shedding, as well as the pattern of fecal shedding over time and the factors affecting this pattern.

Materials and Methods

Challenge model

Calves used in this study were cared for in compliance with the Institutional Animal Care and Use Committee (IACUC) of Cornell University. This randomized, double-blinded study was performed at the College of Veterinary Medicine, Cornell University (Ithaca, NY) from June 2007 through August 2010. Forty-three calves were purchased at birth from a local dairy farm and enrolled in the study as they were born. Control calves (n=7) were enrolled concurrently with test calves (n=36). At least one study author attended all calvings. The perineum of the dam was thoroughly cleaned with povidone-iodine scrub and calves were caught on single-use plastic sheets to prevent on-farm manure contamination. Immediately after birth, a physical examination was performed and an identification tag was placed in the right ear. All calves were fed 2-4L of maternal colostrum or $\geq 100\text{g}$ IgG/dose commercial colostrum replacer (Bovine IgG, Colostrum Replacement, Land O' Lakes Inc.) within the first 4 hours of life. The calves were then transported from the source farm to Cornell University.

At Cornell University, all calves were individually housed in concrete box stalls so as to prevent any contact between calves. Blood samples were collected from each calf within 24-48 hours of life and

the serum total protein was measured in order to assess adequacy of passive transfer. Calves were fed commercial 22% protein/20% fat non-medicated milk replacer (Nursing Formula NT Calf Milk Replacer, Land O'Lakes Inc.) with at least 0.68 kg of dry matter per day, split into 2 feedings, for the duration of the study and water was provided ad libitum. Calves received an oral challenge of *C. parvum* oocysts within the first 24 hours of life. Each calf was inoculated with either 0 oocysts (control) or one of four possible dose magnitudes of a genotyped field strain of *C. parvum* oocysts. At enrollment, both control and test calves were randomized to dose group by a number generator. Seven calves received 0 oocysts, 13 calves received 1×10^3 oocysts, 7 calves received 1×10^4 oocysts, 8 calves received 1×10^5 oocysts, and 8 calves received 1×10^6 oocysts. Study personnel responsible for data collection and analysis were blinded to dose group. Calves were enrolled in the study for 16-21 days.

Control calves (n = 7) were housed in the same facility as test calves, sham dosed, and managed as if they were test calves in order to maintain blinding. Control calves also served as sentinels for cross contamination from test calves, and to help maintain quality assurance in data collection and husbandry practices. To prevent cross contamination, calves were fed and bedded in the same order (youngest to oldest) each day, each calf stall had dedicated equipment and supplies, and all study personnel used single-use personal protective equipment when entering each calf stall.

The oocysts used to dose the calves were purified using a procedure previously described (Jenkins et al., 1997). In brief, feces were collected from naturally infected 6- to 14-day-old calves from a separate commercial dairy operation and processed by continuous-flow differential density flotation. They were stored at 4 °C in suspension with 100 U of penicillin G sodium per ml, 100 mg of streptomycin sulfate per ml, and 0.25 mg of amphotericin B per ml for up to 2 months or until needed. The oocyst DNA was genotyped as *C. parvum* by sequence and restriction fragment length polymorphism (RFLP) analysis via amplification of the small subunit (SSU) rRNA gene in a nested polymerase chain reaction (PCR) as described previously (Jiang et al., 2005). In brief, the primary PCR step amplifies a fragment of approximately 1,325 base pairs, whereas the secondary PCR step results in a fragment of approximately

823 base pairs. In the primary PCR, the following forward and reverse primers were used, respectively: 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCTTCGAAACAGGA-3'. In the secondary PCR, the following forward and reverse primers were used, respectively: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-CTCATAAGGTGCTGAAGGAGTA-3'.

Before inoculation, oocysts were first cleaned for one minute in 0.6% sodium hypochlorite to inactivate viruses and bacteria co-purified with the oocysts, then washed four times with phosphate buffered saline to remove the sodium hypochlorite, quantified using a hemocytometer and finally viability determined using a dye permeability assay as described previously (Campbell et al., 1992; Anguish and Ghiorse, 1997; Jenkins et al., 1997). Viable oocysts were the sum of 49,6-diamidino-2-phenylindole-negative (DAPI-) propidium iodide-negative (PI-) oocysts and DAPI-positive (DAPI+) PI- oocysts; DAPI+ PI+ oocysts were considered inactivated (Jenkins et al. 1999). Oocysts used for dosing were at least 87% viable. Doses were calculated based on the percent viable. Each dose was administered in a 5 ml suspension of *C. parvum* oocysts in reverse osmosis water via the rigid portion of an oroesophageal feeding tube. Followed by 120 ml of water to ensure all of the oocyst suspension was delivered to the calf

Fecal sample analysis

Quantitative analysis of *C. parvum* oocysts in the fecal samples collected was performed using Merifluor Crypto/Giardia immunofluorescence antibody detection reagent from Meridian Diagnostics (Cincinnati, OH) (Xiao and Herd, 1993). The immunofluorescence procedure was modified from the kit instructions. Briefly, a 0.10 g portion of feces was mixed into 10 ml of PBS (pH = 7.4) in a 15 ml conical centrifuge tube. Then, 100 µl of the mixture was removed and 5 µL of Merifluor immunofluorescence antibody reagent was added. The solution was vortexed and incubated in the dark at room temperature for at least 30 min and stored at 4°C until examination. Following incubation, 10.5 µl of the sample was placed on a slide and covered with a coverslip. The 20× objective on a fluorescent compound binocular microscope (460–490 wavelength fluorescent compound binocular microscope Olympus BX41, Olympus America Inc., Center Valley, PA) was used to count the number of oocysts observed. The number of

oocysts observed in 10.5 µl was then multiplied by 10,000 to give the number of oocysts per gram of feces. This count was standardized by the dry weight percentage. Dry weight analysis of fecal samples was obtained by taking a 10 to 20 g portion of each original fecal sample, drying it at 108 °C for a minimum of 24 h (Thermolyne Mechanical Oven, Barnstead International, Dubuque, IA), then weighing it directly (Precision Standard Scale, Ohaus Corporation, Pine Brook, NJ) (Bellosa et al., 2011).

Data Analysis

Data were analyzed using descriptive and inferential methods. For each magnitude of exposure, the probability of shedding on a given day after challenge was estimated using the Kaplan-Meier product limit method (Kaplan and Meier, 1958). Analysis of variance was used to evaluate whether or not there were differences among exposure groups with respect to the onset, cessation, and duration of fecal oocyst shedding and the total amount of fecal oocysts counted/g of feces dry weight that could be attributed to the differences in the 4 exposure groups. The total amount of fecal oocysts counted/g of feces dry weight was normalized via log transformation. Post-hoc analysis of the total amount of fecal oocysts counted/g of feces dry weight across the 4 dose groups was carried out with Tukey's HSD. The relationship between the log-total number of oocysts counted/g of feces dry weight and the log-dose, total protein (g/dl), and number of days until the onset of fecal shedding were evaluated using linear regression methods. The pattern of fecal shedding over time was also evaluated using linear regression methods. Data were analyzed using JMP 9.0 (SAS Institute Inc., 1989-2007).

Results

Among the 43 calves enrolled in the study, 7 were control calves and 36 were experimentally challenged with *Cryptosporidium parvum* oocysts. Calves had a mean serum total protein (g/dl) of 5.3 ± 0.35 (95% CI 5.1 - 5.4). There was no difference in serum total protein (g/dl) among the four dose groups; each group attained adequate passive transfer (>5.0 g/dl). None (0%) of the 7 control calves shed oocysts or had clinical signs consistent with cryptosporidiosis. Thirty-three (91.7%) of the experimentally challenged calves shed *C. parvum* oocysts in their feces, developed diarrhea, and exhibited other clinical

signs associated with cryptosporidiosis. The mean total oocysts counted/g of feces dry weight for all calves that shed oocysts ($n = 33$) was 1.05×10^8 (95% CI 5.03×10^7 - 1.60×10^8).

The average number of days until the onset of fecal oocyst shedding post-challenge was 8.6 ± 1.5 ($n = 33$). Figure 1 shows the probability of onset of fecal oocyst shedding on any given day post-challenge for each dose group. Among the 4 dose groups, the number of days until the onset of fecal oocyst shedding post-challenge tended to be influenced by dose magnitude ($p = 0.17$). Among the 33 calves that shed oocysts in their feces, the earliest onset of shedding was at 4 days post-challenge, and the latest onset of shedding was at 12 days post-challenge (Table 2.1). Both of these individuals were dosed with 1×10^3 oocysts (Figure 2.1). Calves that were experimentally challenged with 1×10^3 and 1×10^4 *C. parvum* oocysts had a median time to onset of fecal shedding post-challenge of 8 days (Table 2.1). Calves that were experimentally challenged with 1×10^5 and 1×10^6 *C. parvum* oocysts had a median time to onset of fecal shedding post-challenge of 7 days (Table 2.1).

	Group A	Group B	Group C	Group D
Onset of Fecal Oocyst Shedding (days)				
<i>mean</i>	9 ± 2	9 ± 1	8 ± 2	8 ± 1
<i>median</i>	8	8	7	7
<i>range</i>	(4 – 12)	(6 – 9)	(5 – 9)	(6 – 9)
Cessation of Fecal Oocyst Shedding (days)				
<i>mean</i>	18 ± 2	17 ± 1	17 ± 2	16 ± 1
<i>median</i>	18.5	17	16.5	16
<i>range</i>	(14 – 21)	(15 – 19)	(14 – 21)	(13 – 16)
Duration of Fecal Oocyst Shedding (days)				
<i>mean</i>	10 ± 2	9 ± 1	10 ± 2	8 ± 2
<i>median</i>	10	9	10	9
<i>range</i>	(5 – 12)	(7 – 11)	(6 – 12)	(5 – 10)
Log-Total Oocysts Counted/g of feces dry weight				
<i>mean</i>	7.4 ± 0.3	8.0 ± 0.3	7.9 ± 0.5	7.7 ± 0.6
<i>median</i>	7.6	7.9	8.1	7.7
<i>range</i>	(7.0 – 7.7)	(7.5 – 8.6)	(7.1 – 8.4)	(6.8 – 8.9)

Table 2.1 The average (mean ± SD), median, and range for the number of days post-challenge until the onset of fecal shedding, the number of days post-challenge until the cessation of fecal shedding, the number of days duration of fecal shedding post-challenge, and the log-total number of oocysts counted/g of feces dry weight in calves experimentally challenged with 1×10^3 (Group A), 1×10^4 (Group B), 1×10^5 (Group C), or 1×10^6 (Group D) oocysts of *C. parvum*

The earliest cessation of fecal shedding was at 13 days post-challenge in a calf dosed with 1×10^6 oocysts, and the latest cessation of fecal shedding was at 21 days post-challenge in 2 calves dosed with 1×10^3 and 1×10^5 oocysts. Similar to the onset of fecal shedding, among the 4 dose groups, the number of days until the cessation of fecal oocyst shedding post-challenge also tended to be influenced by dose magnitude ($p = 0.08$) (Table 2.1). The average duration of fecal oocyst shedding in days was 9.2 ± 1.8 ($n = 33$). There was not a difference in the duration of fecal oocyst shedding across the 4 dose groups ($p = 0.23$).

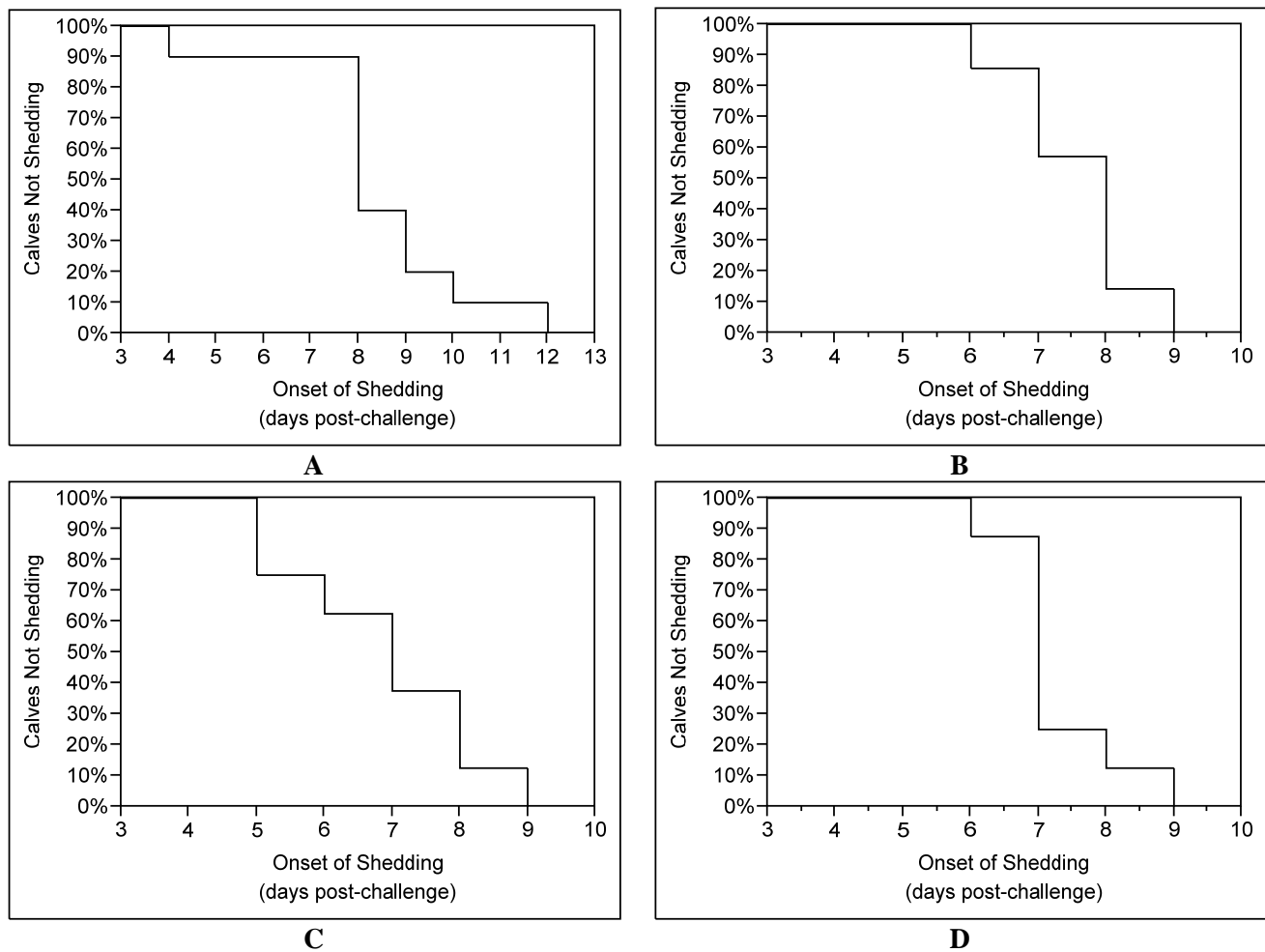


Fig. 2.1 Probability of onset of fecal shedding post-challenge in calves dosed with 1×10^3 (A), 1×10^4 (B), 1×10^5 (C), or 1×10^6 (D) *C. parvum* oocysts

Figure 2.2 shows the distributions of the total amount of oocysts counted per gram of feces among the different dose magnitudes. Higher magnitudes of exposure exhibited more variability in the range of fecal oocyst shedding (Table 2.1). The log-total number of oocysts counted/g of feces dry weight was different across the 4 dose groups ($p = 0.04$) ($n=36$) (Figure 2.2). As shown in Table 2.2, calves in Group A (1×10^3), the lowest magnitude dosing group, tended to have the least amount of total oocysts counted/g of feces dry weight among the four groups; and in post-hoc analysis Group A differed from Group B (1×10^4) ($p = 0.1$), Group C (1×10^5) ($p = 0.09$), and Group D (1×10^6) ($p = 0.15$).

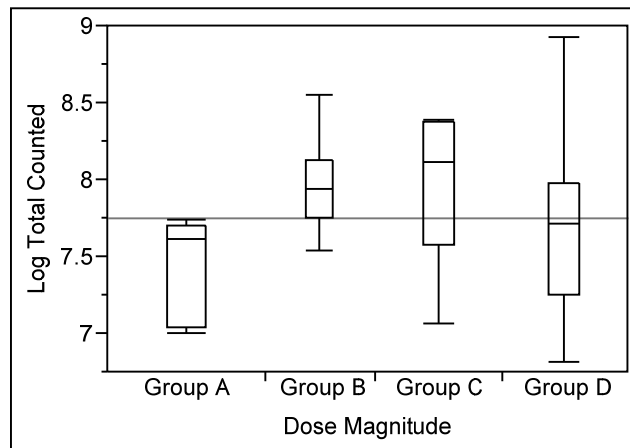


Figure 2.2 The total number of oocysts counted/g of feces dry weight (log-transformed) by dose magnitude [1×10^3 (Group A), 1×10^4 (Group B), 1×10^5 (Group C), or 1×10^6 (Group D)] among calves that shed oocysts (n=33) following experimental challenge with *C. parvum*

Factor	Regression coefficient	SE	p-value
Intercept	10.97	1.63	< 0.0001
Oral exposure (number of oocysts log transformed)	-0.06	0.11	0.62
Onset of fecal shedding (days post-challenge)	-0.28	0.09	0.004
Serum total protein (g/dl)	-0.13	0.28	0.65

Table 2.2 Regression analysis for the log-total amount of oocysts counted for all calves that shed *C. parvum* oocysts after oral challenge

Given the difference in total amount of oocysts shed across the 4 dose groups, we wanted to examine the factors associated with this difference while controlling for serum total protein, the number of days until onset of fecal shedding, and the magnitude of oral exposure. Although the univariate results were suggestive that the log-total amount of oocysts shed is affected by the magnitude of oral exposure, this was not observed ($p = 0.62$) (Table 2.2) when controlling for serum total protein, the number of days until the onset of fecal shedding, and the magnitude of oral exposure. However, there was an inverse relationship between the log-total amount of oocysts counted/g of feces dry weight and the number of days to the onset of fecal shedding ($p = 0.005$). The log-total amount of oocysts counted/g of feces dry weight decreased by 0.28 as the number of days to onset of fecal shedding increased by 1 (Table 2.2).

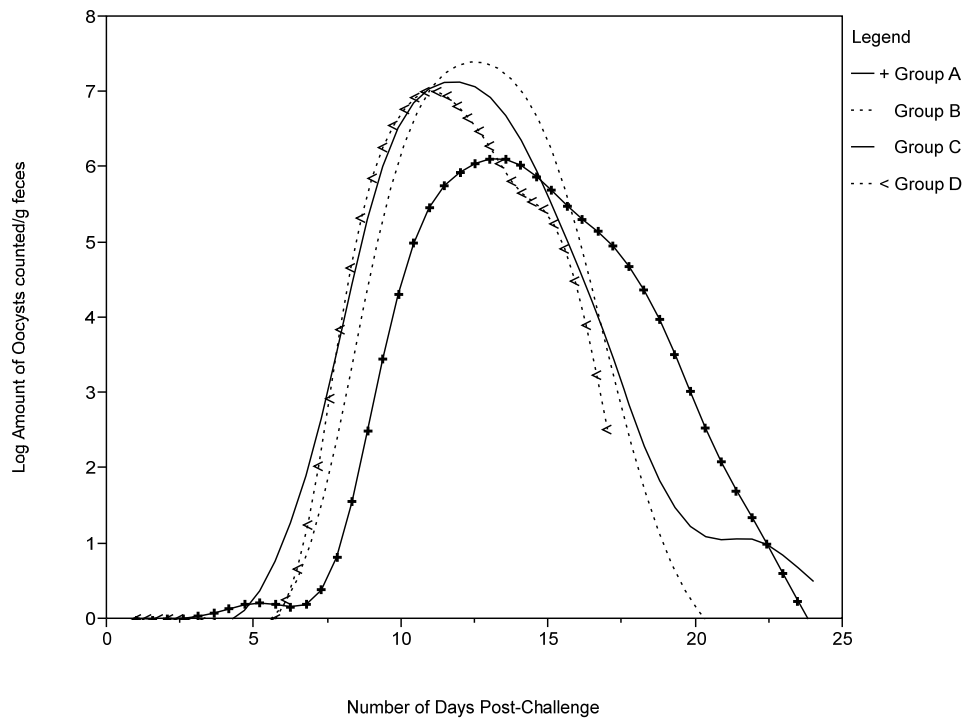


Figure 2.3 The pattern of fecal oocyst shedding over time for each dose group [1×10^3 (Group A), 1×10^4 (Group B), 1×10^5 (Group C), or 1×10^6 (Group D)] among calves that shed oocysts (n=33) following experimental challenge with *C. parvum*

Factor	Regression coefficient	SE	P-value
Intercept	2.2	0.21	< 0.0001
Time	0.11	0.001	< 0.0001
(Time) ²	-0.01	0.0007	< 0.0001

Table 2.3 Regression analysis for the pattern of fecal shedding over time for all calves dosed with *C. parvum* oocysts regardless of dose magnitude

The pattern of fecal shedding over time for all calves exposed to *C. parvum* oocysts, irrespective of dose, was curvilinear (Figure 2.3). The log-total oocysts counted/g of feces dry weight increased with age ($p < 0.0001$), reached a peak, and declined ($p < 0.0001$) (Table 2.3).

Discussion

Cryptosporidium parvum infection is an important agricultural zoonosis (Fayer, 2004). It causes severe illness in people, is associated with increased morbidity and mortality among calves, and can result in economic losses for dairy farms (Waltner-Toews et al., 1986; Warnick et al., 1995; Mor and Tzipori, 2008). In the absence of a commercially available vaccine and consistently effective prophylaxis, the best means by which cryptosporidiosis can be controlled is through preventive measures. Previous studies in people and in calves in field settings suggest that smaller magnitudes of oral exposure reduces the duration of fecal oocyst shedding and may in turn reduce environmental loading, and thus reduce subsequent opportunities for exposure to the parasite (DuPont et al., 1995; Moore et al., 2003).

We have described the relationship between magnitude of oral exposure to *C. parvum*, and the onset, duration, and cessation of fecal shedding. We found a difference in the onset and cessation of fecal oocyst shedding, and in the log-total number of oocysts counted/g of feces dry weight across 4 magnitudes of exposure, but we did not find a difference in the duration of fecal oocyst shedding. In contrast to our study, the study conducted by Moore et al, did find a significant difference in duration of fecal shedding with respect to size of inoculum in dairy calves (Moore et al., 2003). However, in that study 75 calves were enrolled and 29 calves died after day 3 of life. Among the 8 that were necropsied, *Salmonella* spp, rotavirus, and coronavirus were recovered in addition to *C. parvum*. It is therefore possible that many of the calves enrolled in that study experienced a longer duration of shedding due to altered immune function associated with co-infection. In our study, all calves survived through to completion of the study. All calves were tested for infection with *Salmonella* spp, as well as for rotavirus and coronavirus infection, and all were negative, whereby eliminating enteric co-infection as a confounder. In addition to the study by Moore et al., another study conducted by DuPont et al. looking at the infectivity of *C. parvum* in people, suggests that the size of oral inoculum not only influences the duration of fecal shedding, but also influences the onset of fecal shedding (DuPont et al., 1995). However, people in this study with base-line antibody to the parasite experienced more severe clinical illness, which

the authors attribute to increased susceptibility. This may have potentially confounded the onset and duration of fecal shedding. Both of these studies included oral inoculums that were less 1×10^3 oocysts in magnitude, whereas ours did not. Therefore, it is possible that the onset, cessation, and duration of fecal shedding are more appreciably impacted at lower dose magnitudes. It is also possible that the sample size for our study was not large enough to detect this difference.

The median time to onset of fecal shedding in our study was between 7 and 8 days across the 4 dose groups, the earliest onset was 4 days post-challenge, and the latest onset was 12 days post-challenge as is indicated in our survival analysis (Figure 2.1). A paper by Moore et al., reports an overall mean time to onset of fecal shedding across all dose magnitudes of 7.4 days, which is similar to our reported median time. In our study, however, the mean time to onset of fecal shedding was slightly greater than in Moore's study, with the two lowest dose groups having a mean of 9 days, and the two highest dose groups having a mean of 8 days.

With respect to fecal oocyst shedding across the 4 dose groups, 3 experimentally challenged calves never shed oocysts in their feces, 1 calf began shedding oocysts at 4 days post-challenge (Figure 2.1), and another did not begin to shed oocysts until 12 days post-challenge (Figure 2.1). Efforts were made to minimize extreme variability in parasite-level effects by using the same field strain of oocysts that were at least 87% viable. Likewise, efforts were also made to minimize extreme variability in calf-level effects by enrolling calves from the same commercial dairy and ensuring adequate passive transfer of antibodies. These differences are best explained by normal variation in many factors acting in concert with one another including effects attributable to variations in calves, the parasite, and the environment. It should also be noted that among the 3 calves that did not shed, and the 2 calves that shed earliest and latest in the study, all received a dose of 1×10^3 oocysts, which was the lowest dose magnitude we tested. It is conceivable that this was not a large enough infectious dose for the 3 calves that never shed and the 1 calf that did not start to shed until day 12 post-challenge. Given the low infectious dose reported in people, it stands to reason that factors other than magnitude of infectious dose influenced fecal shedding

for these calves (DuPont et al., 1995). These results indicate that the degree of fecal shedding of oocysts is influenced by multiple factors including dose magnitude, parasite lifecycle, parasite-level effects, and calf-level effects. It is also possible that different results might be attained with the use of a different strain, but without testing, we cannot be sure.

The results of this study show that calves exhibit the same pattern of fecal shedding over time regardless of dose magnitude. Calves that receive a lower oral dose begin shedding later and shed fewer total oocysts. This study also demonstrated that calves shed numbers of oocysts that greatly exceeds the magnitude of the dose administered, which is partially attributable to the auto-infective stage of the parasite lifecycle in which sporozoites re-infect the epithelial cells of the intestinal lumen and thus initiate a new cycle of parasite development. While it was not evaluated in this study, it is possible that the auto-infective stage of the parasite life cycle may cause a delay in the onset of fecal shedding at lower infectious doses. Infected calves in turn void readily infective resistant oocysts into the surrounding environment. Given the heartiness of the parasite, the potential for large-scale contamination of the local environment, and the low infectious dose; disease prevention remains to be the best control measure for cryptosporidiosis.

There is an indication that the onset of fecal shedding is influenced by the amount of oocysts to which calves are exposed; calves receiving lower doses of oocysts begin shedding later. Therefore, if onset of fecal oocyst shedding were delayed, environmental parasite loading could be reduced, as well as the risk of exposure for calves and people. Differences in fecal shedding varied across doses and between doses, indicating important effects at both the calf-level and parasite-level. Preventing calf exposure to the parasite entirely is not realistic in conventional calf-rearing systems where the pathogen is often endemic and therefore not an adequate means of disease prevention alone. Instead, an integrated approach to the prevention of cryptosporidiosis in calves should be undertaken in order to address parasite, calf, and environmental factors through the provision of clean and dry housing and an appropriate plane of nutrition. Future studies will be focused on the impact of delayed fecal shedding and reduced dose

magnitudes on fecal oocyst shedding, as well as on delineating the dose-response relationship and determining the ID₅₀ for fecal shedding in dairy calves.

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CHAPTER THREE

***CRYPTOSPORIDIUM PARVUM*: DETERMINATION OF ID₅₀ AND THE DOSE-RESPONSE RELATIONSHIP IN EXPERIMENTALLY CHALLENGED DAIRY CALVES**

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Abstract

The objectives were to determine the median infective dose (ID₅₀) of *Cryptosporidium parvum* and to describe the dose-response relationship including associated clinical illness in experimentally challenged dairy calves. Within the first 24 hours of life, 27 test calves were experimentally challenged with *C. parvum* oocysts and 3 control calves were sham dosed. Test calves received 1 of 8 possible doses (25, 50, 100, 500, 1×10^3 , 1×10^4 , 1×10^5 , and 1×10^6 oocysts). All 27 test calves developed diarrhea. Fecal oocyst shedding occurred in 25 (92.6%) test calves and in 0 control calves. The 2 non-shedding test calves both received 25 oocysts. There was an inverse relationship between dose and time to onset of fecal oocyst shedding ($p = 0.005$). There was no relationship found between dose and duration ($p = 0.2$) or cessation ($p = 0.3$) of fecal oocyst shedding. In addition, there was not a significant relationship between log-dose and the log-peak oocysts ($p = 0.2$) or log-total oocysts ($p = 0.5$) counted/g of feces across the dose groups. There was a positive dose-response relationship between log-dose and diarrhea ($p = 0.01$). However, when controlling for other factors, such as onset and cessation of fecal oocyst shedding, dose was not a significant predictor of diarrhea ($p = 0.5$). Onset and cessation of fecal oocyst shedding were found to be the best predictors of diarrhea ($p = 0.0006$ and $p = 0.04$, respectively). The ID₅₀ for fecal oocyst shedding was 5.8 oocysts, for diarrhea was 9.7 oocysts, and for fecal oocyst shedding with diarrhea was 16.6 oocysts. Given that the ID₅₀ of *C. parvum* is far less than would be excreted into the environment by a naturally infected calf, prevention and control of cryptosporidiosis is a formidable challenge.

Introduction

Cryptosporidium is a genus of apicomplexan protozoal parasites that are globally distributed and are known to infect several species of animals (Fayer, 2004). *Cryptosporidium parvum* and *Cryptosporidium hominis*, which are respectively host-adapted to cattle and people, are recognized as being among the most pathogenic species. Cryptosporidiosis, refers to infection with *Cryptosporidium* spp., and is primarily characterized by villus atrophy and fusion,

intestinal crypt inflammation, and a resultant malabsorptive, maldigestive, osmotic diarrhea. *C. parvum* infection typically occurs in calves less than 1 month old and impacts calf morbidity and mortality (O'Handley et al., 1999). Infected calves shed large numbers of readily infective oocysts in their feces which impacts environmental parasite loading (Nydam et al., 2001).

C. parvum infection is an important zoonosis, causing similar clinical symptoms in people, and is more likely to be acquired by immune suppressed individuals (Anderson, 1998). Transmission is typically via the fecal-oral route. Common risk factors for cryptosporidiosis include ingestion of contaminated water (i.e. fecal accidents in public swimming pools or use of unprotected water sources), poor hygiene, contact with livestock, and failures at municipal water-treatment facilities (MacKenzie et al., 1995; Valderrama et al., 2009; Ng et al., 2012).

There are currently no consistently effective and commercially available treatments or vaccines. Nitazoxanide (NTZ) has shown efficacy in HIV-seronegative patients, but not in HIV-seropositive patients (Amadi et al., 2002). NTZ does reduce fecal oocyst shedding in calves but is not commercially available for use in cattle (Ollivett et al., 2009). Given these challenges, cryptosporidiosis is not only a calf management concern, but is also a global public health concern (O'Handley et al., 1999; Thompson et al., 2008).

The dose-response relationship has been described and median infective dose (ID₅₀) has been determined for some *Cryptosporidium* spp. through investigation carried out in healthy human volunteers. For both *C. parvum* and *C. hominis*, a positive relationship between the size of the inoculum and occurrence of enteric symptoms has been confirmed, thus, the larger the exposure to infective oocysts, the worse the clinical outcome (DuPont et al., 1995; Chappell et al., 1996; Chappell et al., 2006). In people experimentally infected with *C. parvum* and *C. hominis*, 61% developed enteric symptoms (DuPont et al., 1995; Chappell et al., 2006). In human subjects who developed diarrhea and excreted oocysts, the median

infective dose (ID₅₀) for *C. parvum* was determined to be 132 oocysts, and for *C. hominis* was 83 oocysts (DuPont et al., 1995; Chappell et al., 2006).

Similar studies in animals have been restricted to laboratory species. The reported ID₅₀ for *C. parvum* in immunocompetent neonatal mice is 100 – 500 oocysts, and in CD1 neonatal mice is 79 oocysts, which is consistent with the doses reported in people (Ernest et al., 1986; Finch et al., 1993). While experimental infection of livestock is not reported in the literature, studies of natural infection have been conducted. Naturally infected calves experience diarrhea, have a mean onset of fecal oocyst shedding of 16.3 days, and a mean duration of fecal oocyst shedding of 10.5 days (O'Handley et al., 1999). Though not shown conclusively, a study of naturally infected calves suggested that reduced exposure to *C. parvum* oocysts resulted in reduced duration of fecal oocyst shedding (Moore et al., 2003).

Even though studies of natural infection provide important information on disease ecology, they are limited by confounders such as co-infection and lack of experimental controls. Given that *C. parvum* is a known zoonosis, is up to 95% prevalent in US dairy herds, contributes to increased calf mortality, and that there is not an available pharmacologic intervention; it is important to improve our understanding of *C. parvum* epidemiology, through experimental studies of infection, in order to develop appropriate disease control and prevention strategies (Lefay et al., 2001; Trotz-Williams et al., 2008; Tzipori and Widmer, 2008). The objectives of this study were to determine the median infective dose (ID₅₀) of *C. parvum* and to describe the dose-response relationship and associated clinical illness in experimentally challenged dairy calves.

Materials and Methods

Challenge Model

Calf enrollment

Calves used in this study were cared for in compliance with the Cornell University Institutional Animal Care and Use Committee (IACUC). This randomized, double-blinded study was performed at the College of Veterinary Medicine, Cornell University (Ithaca, NY) from February to March 2011. Thirty calves were purchased at birth from a local dairy farm and enrolled in the study as they were born. Control calves (n=3) were enrolled concurrently with test calves (n=30). At least one study author attended all calvings. The perineum of the dam was thoroughly cleaned with povidone-iodine scrub and calves were delivered onto single-use plastic sheets to prevent manure contamination. Immediately after birth, a physical examination was performed and an identification tag was placed in the right ear. All calves were fed 4L of $\geq 50\text{g}$ IgG/L commercial colostrum replacer (Bovine IgG, Colostrum Replacement, Land O' Lakes Inc., St. Paul, MN) within the first 4 hours of life via an oroesophageal feeding tube in order to replicate conventional calf management on commercial dairies as best as possible. Commercial colostrum replacer was fed instead of colostrum in order to minimize variability between calves, to limit potential pathogen exposure, and to provide adequate passive transfer of immunity without providing anti-Cryptosporidium specific antibodies. The calves were then transported from the source farm to Cornell University.

Calf management, sampling, and inoculation

At Cornell University, all calves were housed in a Biosafety Level 2 facility in individual concrete box stalls. Blood samples were collected from each calf within 24-48 hours of life and the serum total protein was measured in order to assess adequacy of passive transfer. Calves

were fed commercial 22% protein/20% fat non-medicated milk replacer (Nursing Formula NT Calf Milk Replacer, Land O'Lakes Inc.) with at least 0.68 kg of dry matter per day, split into 2 feedings, for the duration of the study and water was provided ad libitum. At enrollment, both control and test calves were randomized to a dose group by a number generator. Calves received an oral challenge of *C. parvum* oocysts within the first 24 hours of life. Three calves served as controls and were sham dosed with 0 oocysts. Twenty-seven calves were inoculated with one of eight possible doses of a genotyped field strain of *C. parvum* oocysts. Five calves received 25 oocysts, 4 calves received 50 oocysts, and 3 calves each received 100; 500; 1000; 10,000; 100,000; or 1,000,000 oocysts. All study personnel were blinded to dose group.

Control calves (n = 3) were housed in the same facility as test calves, sham dosed, and managed as if they were test calves in order to maintain blinding. Control calves also served as sentinels for cross contamination from test calves, and to help maintain quality assurance in data collection and husbandry practices. To prevent cross contamination, calves were fed and bedded in the same order (youngest to oldest) each day, each calf stall had dedicated equipment and supplies, and all study personnel used single-use personal protective equipment when entering each calf stall.

A fecal sample was collected from each calf every 24 hours after oral challenge. Clinical data including rectal temperature, general health status, and fecal consistency were recorded every 10-12 hours for each calf. Health status was assessed on a scale of 1 to 4 and fecal consistency was assessed on a scale of 1 to 5 in accordance with previously described methods (Table 3.3.1) (Bellosa et al., 2011). Diarrhea was defined as having at least 2 consecutive fecal consistency scores ≥ 3 . Calves that did not shed oocysts in their feces were enrolled in the study for 21 days. Calves that did have oocysts present in their feces were enrolled in the study until 2 consecutive negative fecal exams were recorded after the onset of fecal shedding. All calves were tested for the presence of rotavirus, coronavirus, and *Salmonella* spp.

The oocysts used to dose the calves were purified using a procedure previously described (Jenkins et al., 1997). In brief, feces were collected from naturally infected 6- to 14-day-old calves from a separate commercial dairy operation and processed by continuous-flow differential density flotation. They were stored until needed at 4 °C in suspension with 100 U of penicillin G sodium per ml, 100 mg of streptomycin sulfate per ml, and 0.25 mg of amphotericin B per ml. The oocyst DNA was genotyped as *C. parvum* by sequence and restriction fragment length polymorphism analysis via amplification of the small subunit (SSU) rRNA gene in a nested polymerase chain reaction (PCR) as described previously (Jiang et al., 2005). In brief, the primary PCR step amplifies a fragment of approximately 1,325 base pairs, whereas the secondary PCR step results in a fragment of approximately 823 base pairs. In the primary PCR, the following forward and reverse primers were used, respectively: 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-3'. In the secondary PCR, the following forward and reverse primers were used, respectively: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-CTCATAAGGTGCTGAAGGAGTA-3'.

Score	Health Status	Fecal Consistency
1	Normal. The calf is alert, hungry, and watches the caretakers. It may stretch when it gets up. The calf will eat greedily, often twitching its tail as it eats.	Normal feces; feces retain form. The feces may be pasty but do not flow across a surface.
2	Mildly depressed. The calf drinks without coaxing, but not aggressively. The calf pays some attention to caretakers and assessment for dehydration (skin tent ≤ 4 seconds, eyes normal) produces equivocal results.	Mild diarrhea; form is a puddle, not a patty. Sufficient water content to slowly flow across or down a surface.
3	Severely depressed. The calf must be coaxed to get up, and has difficulty rising or standing, does not pay attention to caretakers when touched, may refuse to eat, and is clearly dehydrated (e.g., skin tent >9 seconds, separation between eyeball and orbit ≥ 0.5 cm, dry mucous membranes). The calf is unlikely to recover without supportive treatment.	Moderate diarrhea; feces with sufficient water content to easily flow across or down a surface, while leaving some adherent material.
4	Moribund or dead. The calf cannot stand or is dead	Severe diarrhea; part or all of feces are very watery. Feces can drain away leaving little or no residual on a smooth surface (a calf may have very watery feces followed by some solid material and still have severe diarrhea).
5	N/A	Not observed

Table 3.1 Health Status and Fecal Consistency Rubric used for evaluating severity of calf illness and diarrhea

Preparation of inoculum

Before inoculation, oocysts were first cleaned for one minute in 0.6% sodium hypochlorite to inactivate viruses and bacteria co-purified with the oocysts, then washed four times with phosphate buffered saline to remove the sodium hypochlorite, quantified using a hemocytometer and finally viability determined using a dye permeability assay as described previously (Campbell et al., 1992; Anguish and Ghiorse, 1997; Jenkins et al., 1997). Viable oocysts were the sum of 4,6-diamidino-2-phenylindole-negative (DAPI-) propidium iodide-

negative (PI-) oocysts and DAPI-positive (DAPI+) PI- oocysts; DAPI+ PI+ oocysts were considered inactivated (Jenkins et al., 1999). Oocysts used for dosing were at least 87% viable. Doses were calculated based on the percent viable. Each dose was administered in a 5 ml suspension of *C. parvum* oocysts in reverse osmosis water via the rigid portion of an oroesophageal feeding tube. Followed by 120 ml of water to ensure all of the oocyst suspension was delivered to the calf

Fecal sample analysis

Quantitative analysis of *C. parvum* oocysts in the fecal samples collected was performed using Merifluor Crypto/Giardia immunofluorescence antibody detection reagent from Meridian Diagnostics (Cincinnati, OH) (Xiao and Herd, 1993). The immunofluorescence procedure was modified from the kit instructions. Briefly, a 0.10 g portion of feces was mixed into 10 ml of PBS (pH = 7.4) in a 15 ml conical centrifuge tube. Then, 100 µl of the mixture was removed and 5 µL of Merifluor immunofluorescence antibody reagent was added. The solution was vortexed and incubated in the dark at room temperature for at least 30 min and stored at 4°C until examination. Following incubation, 10.5 µl of the sample was placed on a slide and covered with a coverslip. The 20× objective on a fluorescent compound binocular microscope (460–490 wavelength fluorescent compound binocular microscope Olympus BX41, Olympus America Inc., Center Valley, PA) was used to count the number of oocysts observed. The number of oocysts observed in 10.5 µl was then multiplied by 10,000 to give the number of oocysts per gram of feces. This count was standardized by the dry weight percentage. Dry weight analysis of fecal samples was obtained by taking a 10 to 20 g portion of each original fecal sample, drying it at 108 °C for a minimum of 24 h (Thermolyne Mechanical Oven, Barnstead International, Dubuque, IA), then weighing it directly (Precision Standard Scale, Ohaus Corporation, Pine Brook, NJ) (Bellosa et al., 2011).

Data analysis

Data were analyzed using descriptive and inferential methods. Using the Shapiro–Wilk test, data were determined to be non-Gaussian. A Wilcoxon Rank Sum test was used to compare sets of continuous data. For each dose group, the probability of shedding on a given day after challenge was estimated using the Kaplan-Meier product limit method (Kaplan and Meier, 1958). Post-hoc analysis of the percentage of fecal scores ≥ 3 across the dose groups was carried out with Dunn's Post Test. Analysis of variance was used to assess differences in fecal oocyst counts across dose groups. Simple linear regression analysis was used to evaluate the relationship between individual explanatory variables and diarrhea (percent of fecal scores ≥ 3) as well as onset of fecal oocyst shedding. Explanatory variables having $p \leq 0.1$ were selected for analysis using multiple linear regression. Manual backward-stepwise regression was used to remove explanatory variables and their interactions from the model when $p > 0.05$. The ID_{50} for fecal oocyst shedding, diarrhea, and fecal oocyst shedding with diarrhea was estimated using linear regression analysis. The percent of dosed calves that shed oocysts in their stool, developed diarrhea, or both was compared with the total number of calves receiving each dose of oocysts (log transformed) (DuPont et al., 1995). Data were analyzed using JMP 9.0 (SAS Institute Inc., 1989-2007).

Results

Description of study animals

There were 30 calves enrolled in the study; 3 were control calves and 27 were experimentally challenged with *Cryptosporidium parvum* oocysts. Calves were enrolled in the study for 20.5 ± 1.1 days, during which time the mean number of fecal scores recorded was 38.2 ± 2.7 and the mean number of health scores recorded was 40.9 ± 2.5 . Across all groups, the mean serum total protein measurement (g/dl) was 4.8 ± 0.4 (95% CI 4.6 – 4.9) and there were no differences between groups ($p = 1.0$). None of the calves tested positive for coinfection with rotavirus, coronavirus, or *Salmonella* spp.

Description of fecal oocyst shedding

None of the 3 control calves developed diarrhea or fecal oocyst shedding. All 27 experimentally challenged calves developed diarrhea (at least 2 consecutive fecal consistency scores ≥ 3). Twenty-five calves shed *C. parvum* oocysts in their feces and 2 calves did not. The 2 non-shedding calves were both challenged with 25 oocysts. The mean time to onset of fecal oocyst shedding was 5.6 ± 2 days (95% CI 4.8 – 6.5) (n = 25) (Table 3.2).

Dose of <i>C. parvum</i> oocysts		25	50	100	500	1,000	1 x 10 ⁴	1 x 10 ⁵	1 x 10 ⁶
Onset of fecal oocyst shedding (days)	mean	7.3	8.8	5.7	5.3	4.7	4.7	5	2.7
	median	7	9	6	5	4	4	5	3
	range	(7 – 8)	(7 – 10)	(5 – 6)	(5 – 6)	(4 – 6)	(4 – 5)	(4 – 6)	(2 – 3)
Duration of fecal oocyst shedding (days)	mean	11	9.5	11.7	9.7	10.7	13.3	10.7	13
	median	11	9	12	9	11	13	9	12
	range	-	(9 – 11)	(11 – 12)	(8 – 12)	(9 – 12)	(13 – 14)	(8 – 15)	(9 – 18)
Cessation of fecal oocyst shedding (days)	mean	18	18.3	17.3	15.3	15.3	18	15.7	15.7
	median	18	18.5	18	15	15	18	14	14
	range	(17 – 19)	(16 – 20)	(16 – 18)	(14 – 17)	(15 – 16)	(17 – 19)	(14 – 19)	(12 – 21)

Table 3.2 The mean, median, and range for the number of days post-challenge until the onset of fecal shedding, the number of days duration of fecal shedding post-challenge, and the number of days post-challenge until the cessation of fecal shedding, in calves experimentally challenged with 25, 50, 100, 500, 1 x 10³, 1 x 10⁴, 1 x 10⁵, or 1 x 10⁶ oocysts of *C. parvum*

There was an inverse relationship between dose and time to onset of fecal oocyst shedding, i.e., calves that received smaller doses began shedding later (p = 0.005). As the log-dose increased by 1, the

time to onset of fecal oocyst shedding was shortened by 1 day ($p < 0.0001$) (Figure 3.1a). Between dose groups, calves that received 25 or 50 oocysts shed later than calves that received 1,000,000 oocysts ($p = 0.06$ and $p = 0.005$, respectively) (Figure 3.2). The mean duration of fecal oocyst shedding was 11.1 ± 2.4 days (95% CI 10.1 – 12.1) ($n = 25$) (Table 3.2). The mean time to cessation of fecal oocyst shedding was 16.8 ± 2.2 days (95% CI 15.8 – 17.7) ($n = 25$) (Table 3.2). Dose did not influence duration of fecal oocyst shedding ($p = 0.2$) or cessation of fecal oocyst shedding ($p = 0.3$). However, at higher doses more variability was observed in both duration and cessation of fecal oocyst shedding (Table 3.2).

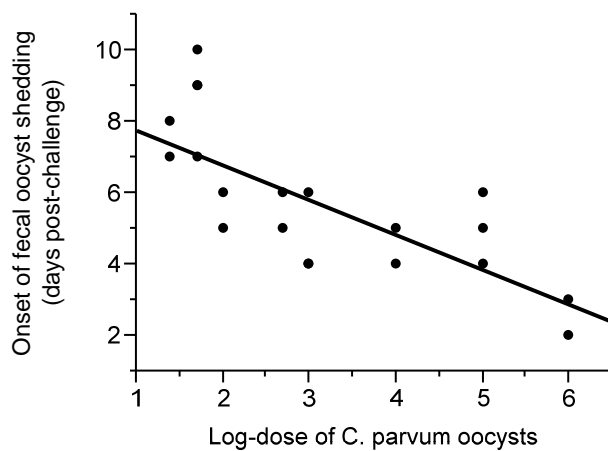


Figure 3.1a The relationship between log-dose and the onset of fecal oocyst shedding in calves experimentally challenged with *C. parvum* oocysts

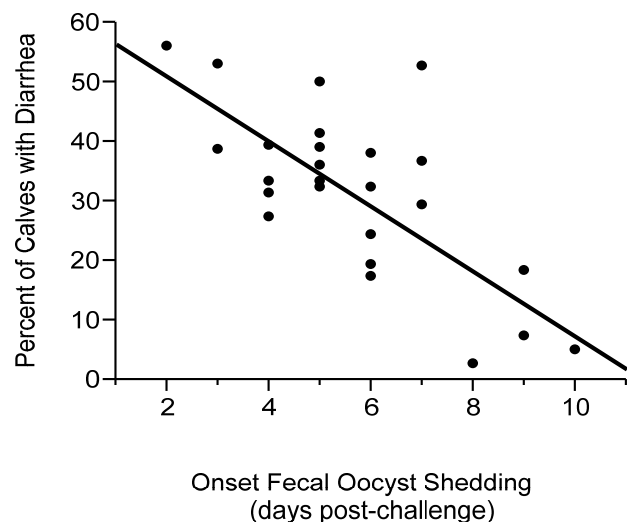


Figure 3.1b The relationship between the onset of fecal oocyst shedding and the percent of calves with *C. parvum* oocysts

When the dose groups are collapsed into 3 dose levels (25 and 50 oocysts, 100 – 100,000 oocysts, and 1,000,000 oocysts) there is a difference in time to onset across the 3 levels ($p < 0.0001$). Between dose levels, calves that received 25 and 50 oocysts shed later than calves that received 100 – 100,000 and 1,000,000 oocysts ($p = 0.003$ and $p = 0.0002$, respectively). Figure 3.2 shows the probability of onset of fecal oocyst shedding on any given day post-challenge for the 3 dose levels.

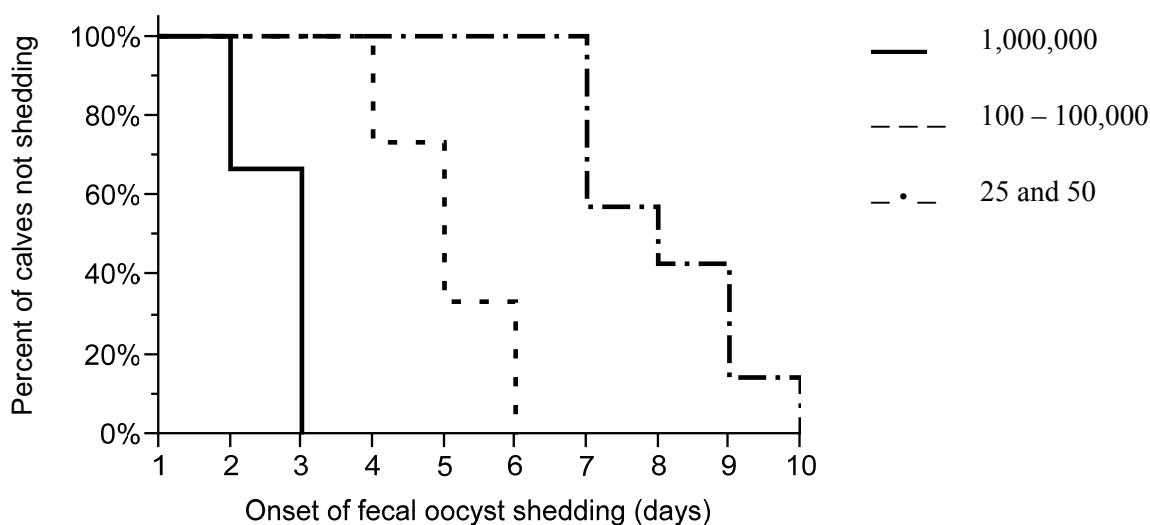


Figure 3.2 Probability of onset of fecal shedding post-challenge in calves dosed with 25 and 50, 100 – 100,000, and 1,000,000 *C. parvum* oocysts

Fecal oocyst shedding and diarrhea

Fecal shedding and diarrhea occurred at all doses. There was an inverse relationship between onset of fecal oocyst shedding and diarrhea, i.e., calves that began to shed oocysts sooner after dosing experienced more days of diarrhea ($p < 0.0001$) (Figure 3.1b). The log-peak oocysts counted was 7.8 ± 0.4 (95% CI 7.6 – 8.0) and the log-total oocysts counted was 7.9 ± 0.4 (95% CI 7.7 – 8.1). There was no difference in log-peak or log-total oocysts counted/g of feces across the dose groups ($p = 0.2$ and $p = 0.5$, respectively) or among the 3 dose levels ($p = 0.3$ and $p = 0.9$, respectively). Among the 5 calves dosed with 25 oocysts, 3 calves shed oocysts, 2 of which also developed diarrhea. For the remaining doses, all calves shed and developed diarrhea (Table 3.3). Health scores > 1 occurred in 2 calves, but only occurred once for each calf. Calf 207 had health scores > 1 for several consecutive days. This calf had a septic stifle joint secondary to an umbilical abscess, and was excluded from health score analysis, but included in all other analysis. The explanatory variables log-dose, total protein on day 2 of enrollment, weight on day 5 of enrollment, weight on the final day of enrollment, onset of fecal oocyst shedding, duration of fecal oocyst shedding, cessation of fecal oocyst shedding, log-peak number of fecal oocysts counted, and log-total

number of fecal oocysts counted were all evaluated via simple linear regression to evaluate a possible relationship with the diarrhea (Table 3.4). The variables log-dose, weight on final day of enrollment, onset of fecal oocyst shedding, and cessation of fecal oocyst shedding all had P-values ≤ 0.1 and were retained for analysis via multiple linear regression. In the final model, onset and cessation of fecal oocyst shedding were found to be the best predictors of diarrhea ($p = 0.0006$ and $p = 0.04$, respectively) (Table 3.5). Simple linear regression was also conducted using the same explanatory variables to evaluate the relationship with onset of fecal oocyst shedding (Table 3.4). Log-dose, duration of fecal oocyst shedding, and cessation of fecal oocyst shedding were found to have p-values ≤ 0.1 and were retained for analysis via multiple linear regression. The best predictors of onset of fecal oocyst shedding were duration and cessation of fecal oocyst shedding ($p < 0.0001$ for both predictors) (Table 3.5).

Dose of <i>C. parvum</i> oocysts	Log Dose of <i>C. parvum</i> oocysts	No. Calves Challenged	Fecal Shedding (%)	Diarrhea (%)	Fecal Shedding + Diarrhea (%)
0	0	3	0 (0)	0 (0)	0 (0)
25	1.4	5	3(60)	3 (60)	2 (40)
50	1.7	4	4 (100)	3 (75)	3 (75)
100	2	3	3 (100)	3 (100)	3 (100)
500	2.7	3	3 (100)	3 (100)	3 (100)
1,000	3	3	3 (100)	3 (100)	3 (100)
10,000	4	3	3 (100)	3 (100)	3 (100)
100,000	5	3	3 (100)	3 (100)	3 (100)
1,000,000	6	3	3 (100)	3 (100)	3 (100)

Table 3.3 Risk of fecal oocyst shedding, diarrhea, and fecal oocyst shedding with diarrhea in calves experimentally challenged with *C. parvum* oocysts.*

* Linear regression analysis of the data yielded and ID₅₀ of 5.8 oocysts for fecal oocyst shedding, 9.7 oocysts for diarrhea, and 16.6 oocysts for fecal oocyst shedding with diarrhea

Explanatory Variable	Outcome = Diarrhea			Outcome = Onset of Oocyst Shedding		
	B ₀ and B ₁	SE	P - value	B ₀ and B ₁	SE	p - value
Diarrhea						
<i>intercept</i>	-	-	-	8.8	0.7	<0.0001
<i>regression coefficient</i>				-0.1	0.02	<0.0001*
Onset of fecal oocyst shedding						
<i>intercept</i>	61.0	6.3	<0.0001	-	-	-
<i>regression coefficient</i>	-5.2	1.1	<0.0001*			
Log Dose						
<i>intercept</i>	10.5	4.5	0.03	8.7	0.6	<0.0001
<i>regression coefficient</i>	6.2	1.4	0.0001*	-1.0	0.2	<0.0001*
Total Protein on Day 2						
<i>intercept</i>	67.4	36.0	0.07	5.2	6.1	0.4
<i>regression coefficient</i>	-8.4	7.5	0.3	0.09	1.3	0.9
Weight on Day 5						
<i>intercept</i>	3.9	17.3	0.8	9.5	2.5	0.0009
<i>regression coefficient</i>	0.2	0.2	0.2	-0.04	0.02	0.1*
Weight at Study Completion						
<i>intercept</i>	-6.6	17.9	0.7	10.0	2.7	0.001
<i>regression coefficient</i>	0.3	0.2	0.06*	-0.04	0.02	0.1*
Duration of Fecal Oocyst Shedding						
<i>intercept</i>	28.6	14.4	0.06	9.8	1.8	<0.0001
<i>regression coefficient</i>	0.3	1.3	0.8	-0.4	0.2	0.02*
Cessation of Fecal Oocyst Shedding						
<i>intercept</i>	91.2	18.7	<0.0001	-0.18	2.9	1.0
<i>regression coefficient</i>	-3.5	1.1	0.004*	0.3	0.2	0.05*
Log Peak Oocysts Counted						
<i>intercept</i>	-13.4	51.8	0.8	17.4	6.9	0.02
<i>regression coefficient</i>	5.8	6.6	0.4	-1.5	0.9	0.1*
Log Total Oocysts Counted						
<i>intercept</i>	-38.5	52.3	0.5	13.5	7.3	0.08
<i>regression coefficient</i>	8.9	6.6	0.2	-1.0	0.9	0.3

Table 3.4 Simple linear regression analysis evaluating the relationship between individual explanatory variables and two possible outcomes, diarrhea and the onset of fecal oocyst shedding.

*Explanatory variables with a p – value ≤ 1.0 were selected for inclusion in the multiple linear regression model

Model 1, Y = Diarrhea*				Model 2, Y = Days to Onset of Oocyst Shedding			
Explanatory Variable	Regression Coefficient	SE	p - value	Explanatory Variable	Regression Coefficient	SE	p - value
Intercept	90.5	14.5	< 0.0001	Intercept	-0.3	0.5	0.6
Onset of fecal shedding	-4.3	1.1	0.0006	Duration of fecal shedding	-1.0	0.03	<0.0001
Cessation of fecal shedding	-2.1	0.9	0.04	Cessation of fecal shedding	1.0	0.04	<0.0001

Table 3.5 Final models following manual backward stepwise regression to analyze the relationship between the explanatory variables onset, cessation, and duration of fecal oocyst shedding and the outcome variables diarrhea and onset of fecal oocyst shedding.

* Diarrhea is defined as 2 or more consecutive feedings with a fecal score ≥ 3

Determination of ID₅₀

There was a positive relationship between log-dose and fecal oocyst shedding with diarrhea ($p = 0.01$) (Figure 3.3) as well as between log-dose and diarrhea alone ($p = 0.007$). The ID₅₀ for shedding was 5.8 oocysts, for diarrhea was 9.7 oocysts, and for shedding and diarrhea was 16.6 oocysts (Table 3.6).

Factor		SE	p - value	R Square	ID ₅₀ (oocysts)	
Shedding						
	<i>intercept</i>	114	9.5	<0.0001	0.45	5.8
	<i>regression coefficient</i>	-48.8	22.0	0.07		
Diarrhea						
	<i>intercept</i>	118.2	7.6	<0.0001	0.71	9.7
	<i>regression coefficient</i>	-67.4	17.5	0.009		
Shedding and Diarrhea						
	<i>intercept</i>	125.2	11.2	<0.0001	0.67	16.6
	<i>regression coefficient</i>	-91.8	26.1	0.01		

Table 3.6 Regression analysis to determine the ID₅₀ for fecal oocyst shedding, diarrhea, and fecal oocyst shedding with diarrhea in calves experimentally challenged with *C. parvum* oocysts

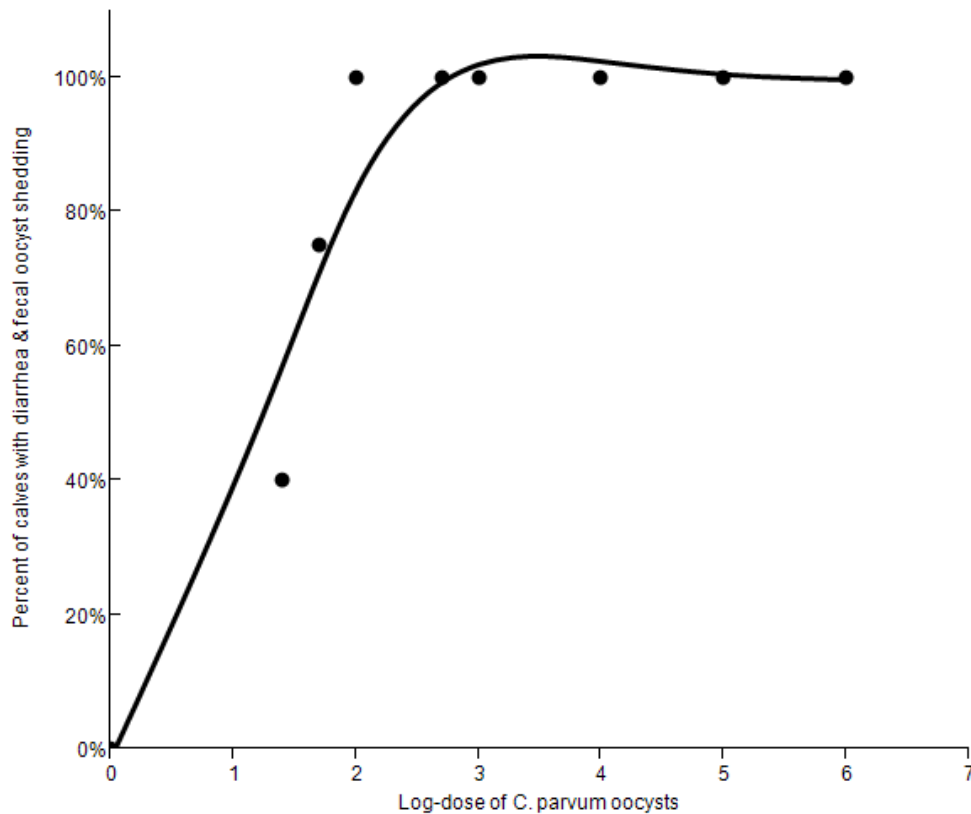


Figure 3.3 The relationship between dose of *C. parvum* oocysts and the percent of calves with diarrhea and fecal oocyst shedding

Discussion

In this study we have demonstrated a positive dose-response relationship between *C. parvum* infection and fecal oocyst shedding with diarrhea. We have determined that in neonatal dairy calves the ID₅₀ of *C. parvum* is 16.6 oocysts, depending on which clinical definition of infection is applied. This dose is orders of magnitude less than number of oocysts typically shed into the environment by naturally infected calves, which has been reported to be in excess of 3×10^{10} oocysts in a calf that sheds oocysts for 6 days (Nydam et al., 2001). In our current study, calves that received the lowest doses of *C. parvum* oocysts began fecal oocyst shedding later, and this was associated with fewer days of diarrhea. Given that *C. parvum* is prevalent in dairy calves, ubiquitous in the environment, and very difficult to kill, its control poses a challenge to dairy farmers (Fayer et al., 1997; Trotz-Williams et al., 2005; Trotz-Williams

et al., 2007). In the fecal-oral transmission cycle, knowing that small levels of exposure can result in a large amount of fecal oocyst shedding, and not knowing exactly how many oocysts a calf is exposed to on any given day, poses the greatest challenge for control. However, it is very likely that calf exposure greatly exceeds the ID₅₀ we have reported. Therefore, findings from this study suggest that unless *C. parvum* is completely eliminated from the environment, it will be very difficult to reduce the incidence of cryptosporidiosis.

Previous studies report a mean onset of fecal oocyst shedding of 7.4 days in experimentally challenged calves, and 16.3 days in naturally infected calves (O'Handley et al., 1999; Moore et al., 2003). A study of healthy human volunteers reported an average onset of fecal oocyst shedding of 9 days (DuPont et al., 1995). We found an inverse relationship between onset of fecal oocyst shedding and dose. Calves that received smaller doses began shedding later, which was also found in a prior study (Zambriski et al., 2013). Likewise, a DuPont et al. (1995) report a relationship between onset of fecal oocyst shedding in people and size of oral inoculum, i.e., at higher doses, infection tended to occur sooner and last longer. Other studies in calves and people have reported different relationships between duration of fecal oocyst shedding and dose (DuPont et al., 1995; Moore et al., 2003). In a study by Moore et al. (2003), an association between dose and duration of fecal oocyst shedding is reported. In that study, 32 of 75 calves died, and among the 8 calves necropsied, all were found to have co-infection with *Salmonella spp*, rotavirus, and coronavirus, in addition to *C. parvum*. Therefore, it is possible that the relationship reported between dose and duration of fecal shedding was influenced by co-infection. In the study conducted in healthy human volunteers challenged with *C. parvum*, a non-statistically significant relationship was reported between dose and duration of oocyst excretion, but the authors do not discuss possible reasons for this association. With respect to enteric symptoms, Moore et al. (2003) report an inverse relationship between onset of diarrhea and duration of diarrhea in calves, and DuPont et al. (1995) report a relationship between dose and occurrence of enteric signs. Our study found a positive relationship between dose and diarrhea, but in the final model, onset and cessation of fecal oocyst shedding were

found to be better predictors of diarrhea. This can be explained in part by the parasite life cycle and associated pathology of the gastrointestinal (GI) tract. Calves that shed sooner or longer, may be more likely to experience a longer auto-infective stage, or may experience more and recurrent villus damage over time, both of which could result in a longer time to recovery and healing of the GI tract and a longer and more severe episode of diarrhea.

Depending on the definition of infection applied, the ID₅₀ of *C. parvum* in calves in our study ranged from 5.8 to 16.6 oocysts. To our knowledge, this is the first study to report this information in calves. In other species, the reported ID₅₀ of *C. parvum* is also very low. In outbred neonatal CD1 mice it is 79 oocysts, and is as low as 60 oocysts in wild-type neonatal mice (Finch et al., 1993; DuPont et al., 1995). Immunosuppressed Mongolian gerbils become infected when challenged with 100 *C. parvum* oocysts (Baishanbo et al., 2005). In healthy human volunteers, the ID₅₀ is reported to be 132 oocysts, but infections occurred at all dose levels, including 30 oocysts (DuPont et al., 1995). For infection with *C. hominis* in healthy human volunteers, the ID₅₀ is estimated to be 10-83 oocysts (Chappell et al., 2006). Our study findings are consistent with those reported by other researchers, and again illustrate the point that the dose of *C. parvum* oocysts required to induce illness and fecal oocyst shedding is relatively low compared to the environmental load.

Since this study was a controlled experimental trial, our study calves were maintained in clean dry housing, received appropriate dry matter intake, were not challenged with other GI parasites, bacteria, or viruses, and may therefore be considered to be in better overall health than the general population of calves on North American dairy farms. Thus, when this research is translated to field settings, it is possible that it is an overestimation of ID₅₀ and the dose-response relationship, and that more severe clinical outcomes could occur. Given that none of the control calves developed diarrhea or fecal oocyst shedding, it is unlikely that cross contamination occurred between challenged calves or that the challenged calves were exposed to oocysts in any quantity greater than their initial challenge dose.

This study demonstrates that calves are susceptible to *C. parvum* infection at very low doses, and will experience clinical illness and fecal oocyst shedding. The degree of fecal oocyst shedding associated with cryptosporidiosis is dramatically disproportionate to the ID₅₀ of *C. parvum*, which contributes to environmental loading and infection of subsequent calves. This study showed that calves receiving a lower dose began to shed oocysts later, and this was associated with less diarrhea. However, while these calves fared better from a clinical perspective, there was no difference in the log-total or log-peak oocysts counted/g of feces across all doses, meaning that regardless of the level of exposure, the degree of environment loading is unaffected. Therefore, the best method of controlling cryptosporidiosis is to prevent exposure entirely, however, in settings where this is not feasible, keeping exposed calves clean, dry, and nourished will minimize the impact of disease on calf health and wellbeing (Ollivett et al., 2012).

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CHAPTER FOUR

ASSOCIATION OF LIVESTOCK MANAGEMENT AND HOUSEHOLD LOCATION WITH HISTORY OF DIARRHEA AMONG AGRO-PASTORALISTS IN THE AMHARA REGION OF ETHIOPIA

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Abstract

The objectives were to describe common animal husbandry and manure management activities and the associated division of animal husbandry labor by age and sex, and to determine the risk factors associated with reported history of diarrhea among Ethiopian agro-pastoralists in the Amhara Region. A semi-structured and standardized household survey with both closed and open ended questions was administered at 69 households in urban, peri-urban, and rural settings near Bahir Dar, Ethiopia. Within these 3 strata, households were purposively and conveniently sampled. Households that did not have children < 5 years and did not have livestock < 3 months old were excluded. Data were analyzed using descriptive and inferential methods, as well as multivariable logistic regression. The predominant husbandry system was semi-intensive (51%). In 75% of households, women were primarily responsible for manure management. Compared to urban households and controlling for other factors, the odds of being engaged in animal husbandry were 30 times greater for children < 5 living in peri-urban households (95% CI 5.0 - 594; $p < 0.001$) and 8 times greater for children < 5 living in rural households (95% CI 1.1 - 167; $p = 0.04$). There was not an association between history of diarrhea in the previous 2 weeks in people and history of diarrhea in the previous 2 weeks in livestock (OR 3, 95% CI 0.7 – 13, $p = 0.18$), but there was an association between history of diarrhea in the previous 2 weeks in people and type of animal husbandry system. The odds of reporting diarrhea in the previous 2 weeks in households that intensively managed their livestock were 2.4×10^7 times greater than for households that practiced extensive management. However, in the multivariable regression analysis, household setting was the best predictor of diarrheal history in the previous 2 weeks ($p = 0.0003$); the odds were 5.7×10^7 times greater for rural households than urban households (7.7 – NC; $p < 0.0001$). Our findings indicate that household location is a more important predictor of diarrheal disease history than livestock diarrheal history and husbandry system.

Introduction

In the 2008 update on the Global Burden of Disease, the World Health Organization reported that diarrhea was the second leading cause of disease burden worldwide across all ages and accounted for 72.8 million years of life lost (disability-adjusted life year, DALY) in 2004 (WHO, 2008). In children under 5 years of age, diarrhea not only contributes to reduced DALYs, it is the second leading cause of death worldwide (WHO, 2008). On average, in the first 5 years of life, every child will contract diarrhea several times, with the potential for severe dehydration, nutritional impairment, stunting, wasting, and death (Bern and Glass, 1994; Tumwine et al., 2003; Eppig et al., 2010). In addition to these unfavorable outcomes, frequent episodes of diarrhea in the first 2 years have been negatively correlated with cognitive development and early school performance (Guerrant et al., 1999; Niehaus et al., 2002). Thus, diarrhea contributes to impaired wellbeing and suffering and its impact may be more sustained than initially perceived, factoring into diminished human capital and persistent poverty.

Given the impact of diarrheal disease worldwide, it is important to understand the epidemiology of diarrheal disease transmission in order to help reduce the global disease burden. Diarrhea worldwide is most often associated with infectious causes, including zoonotic diseases, which are transmitted between people and animals. Zoonotic enteric pathogens such as *Salmonella* spp, *Campylobacter* spp, *Escherichia coli*, *Cryptosporidium* and *Giardia* are frequent causes of diarrheal disease (Acha and Szyfres, 2003). Contact with livestock and their manure is a commonly reported risk factor associated with transmission of these pathogens and with zoonotic diarrheal illness (Grados et al., 1988; Adah et al., 2002; Fayer, 2004; Rao Ajjampur et al., 2007; Coles et al., 2009; Febriani et al., 2009). Compared to developed countries, contact with livestock is more frequent in developing countries where people live in close proximity to livestock and are dependent upon them for livelihoods.

Having approximately 74 million animals, Ethiopia's livestock population is the largest on the African continent and accounts for 30-35% of the agricultural gross domestic product (GDP) (Benin et al., 2003). Livestock farming is estimated to provide livelihood for 65% of Ethiopians and more than 85%

of farm cash income (Food and Agriculture Organization of the United Nations, 1999; Benin et al., 2003). As such, livestock play an important role in poverty prevention and food security in the region. However, livestock-keeping requires labor inputs and can increase risk of zoonotic disease transmission between livestock and livestock-keepers. Livestock husbandry and management systems in Ethiopia are varied and include pastoral, agro-pastoral, and cooperative management systems (co-op). Co-ops, in which farmers pool resources such as land and equipment and share profits, are much less common but do function efficiently and have a presence in the agricultural community. In northern Ethiopia, and in the Amhara region specifically, agro-pastoral systems predominate.

Mixed crop-livestock, or agro-pastoralist systems, are semi-intensive in nature, but the level of intensification can vary. Agro-pastoralist livestock keepers, by definition, derive more than 50% of household gross revenue from farming and 10-50% from livestock (Swift, 1988). Provision of feed and water to livestock is most often extensive in nature, but manure management is often intensive and dictated by household demands for fuel, compost, and home construction.

While there are reports documenting contact with livestock and manure as risk factors for diarrheal disease, there are no reports that describe the nature and extent of this contact with respect to husbandry and manure management. For example, milking cows, herding cattle to a water point, and collecting diarrheic manure for compost all constitute “contact with livestock” but may not be equivalent activities from the standpoint of disease transmission risk. Recently, Kimani et al. found that social and gender factors were important determinants of exposure to zoonotic disease, but this was in the context of urban dairying in Kenya among adult populations (Kimani et al., 2012). Husbandry and manure management systems require varying levels of labor input and animal contact, and the division of household labor often varies with age and sex, and can extend to include children under 5 years of age, as well as adolescents and adults. Therefore, the objectives of this study were to: 1) Describe common animal husbandry and manure management activities and the associated division of animal husbandry

labor by age and sex, and 2) Determine the risk factors associated with reported history of diarrhea among Ethiopian agro-pastoralists in the Amhara Region.

Materials and Methods

Study design

An epidemiologic cohort study was performed between February and April 2011 in the Amhara Region of Ethiopia. Livestock farmers living in urban, peri-urban, and rural areas near Bahir Dar, Ethiopia were identified in collaboration with The Donkey Sanctuary and The Amhara Region Government Veterinary Service (ARGVS). The Donkey Sanctuary is an international non-governmental organization focused on improvement of health and welfare of donkeys in developing countries through provision of free medical services and community outreach and education. Both The Donkey Sanctuary and the ARGVS have a long history of positive rapport with area livestock-keepers, and the veterinarians and veterinary technicians are trusted community members. We partnered with these individuals in order to gain access to the study population, establish trust with study participants, and to minimize bias in data collected via household surveys. Furthermore, it was important to us to partner with individuals and organizations affiliated with livestock health as a means of gaining insights specific to animal husbandry that might provide perspective on the desirability, feasibility, and subsequent adoption of interventions.

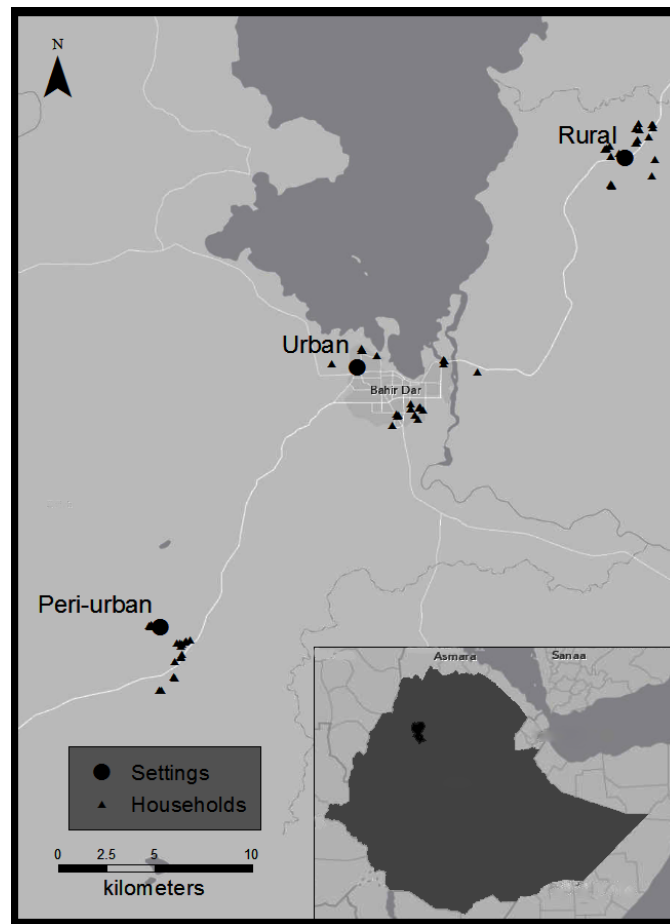


Fig. 4.1 Map of study sites, Bahir Dar, Ethiopia

Study site and participants

The study was conducted in the Amhara Region of Ethiopia. Situated in northern Ethiopia, and bordered by South Sudan, the population is approximately 17 million people (Population Census Commission, 2007). The region is relatively homogeneous with respect to ethnicity and religion; 91.5% of the population is ethnically Amharic and speaks Amharic as their primary language, 82.5% of the population is Orthodox Christian, and 17.2% are Muslim (Population Census Commission, 2007). Agriculture is reported to be the primary source of household income for 64% of women and 80% of men (DHS, 2011). The prevalence of diarrheal disease in children < 5 in the two weeks immediately prior to Demographic Health Survey administration was 13.7% in the Amhara Region, which is comparable to the

national average of 13% (DHS, 2011). The prevalence of HIV in the region is 1.6% and, like diarrheal prevalence, only slightly exceeds the national average which is 1.5% (DHS, 2011).

Agricultural activities in the area are predominantly mixed-crop livestock or agro-pastoral systems. Unlike the pastoralists of southern Ethiopia who are nomadic, agro-pastoralists in the Amhara Region are settled and maintain a homestead. Livestock are herded daily to graze and drink, though in some cases, feed and water are collected and carried to the homestead for livestock consumption. All livestock taken to graze return to the homestead each day and are housed at the homestead overnight. There is a weekly livestock market where cattle are sold to livestock traders, typically for transport to Addis Ababa, but also for local consumption. Similarly, there is a more frequent market (2-3 times/week) for sheep. Goats are not typically kept as they tend to feed on the region's main cash crop, *chat* (also referred to as *qat* or *khat*). *Chat* leaves, which are commonly chewed by men, contain an amphetamine-like stimulant that is mildly to moderately addictive. Other important crops grown in the region include teff and millet. Most households rear poultry, and some households engage in traditional beekeeping for the production of honey. In these households, the women raise the poultry and the men attend to the beehives. Also, given the proximity of Lake Tana, the largest inland body of water in Ethiopia and the source of the Blue Nile, some households engage in activities such as fishing.

Kinship is patrilocal and patrilineal. Marriage is typically monogamous, but polygamy is practiced, despite having been outlawed. At the household level, it is common to have 2 or 3 generations residing in one main structure. The household structure is typically 100 to 150 square feet in total area, which may be divided into 2 rooms. The walls are framed with wood and filled with mud and manure, which is also used to construct the floors, seating, and shelving. It is a longstanding practice that livestock sleep inside the home with the family at night, though some exceptions do exist.

Sampling strategy

Community Animal Health Workers affiliated with ARGVS and who had a strong relationship with the local community helped to identify households for possible enrollment in the study. Farmers

residing in urban, peri-urban, and rural settings, relative to Bahir Dar, were selected to participate in the study through convenience and purposive sampling measures with efforts made to minimize selection bias. These sampling methods were most appropriate due to the paucity of background data, baselines, and sampling frames, as well as for the desire to target households that had members under 5 years of age. Efforts were made to sample equally among the 3 geographic regions and to attain representative samples, accounting for both the number of farms in the region as well as sub-regions arising from either geographic or municipal partitions. This technique, known as sampling until saturation, is often applied when trying to characterize a system within a sample population that is relatively homogenous, and therefore, each additional interview in a similar area returns less and less new information (Guest et al., 2006; Havas et al., 2012). By conducting surveys in this fashion, the ability to identify trends in livestock husbandry and management is preserved, specifically in cases when a more traditional formal survey methodology is impossible to conduct (Marshall, 1996; Guest et al., 2006).

Households that did not have at least 1 child under 5 and livestock under 3 months of age were excluded. Two households satisfied all of the inclusion criteria but were excluded due to perceived bias and lack of confidence in the truthfulness of the answers provided.

Household setting (urban, peri-urban, and rural) was defined in part by the traditionally accepted definition pertaining to physical distance from an urban hub, but was further defined using multiple factors relating to access to resources. Households were categorized as “urban” if they were in close proximity ($\leq 5\text{km}$) to the center of Bahir Dar, the majority of roads were paved, and there was frequent reliable access (several daily routes) to public transit and other amenities (such as local businesses, government offices, and hospitals and health clinics). Households were categorized as “peri-urban” if they were located $> 5\text{km}$ from Bahir Dar city center, there was a mixture of paved and unpaved access roads, public transit to Bahir Dar city center and other amenities was available 2-3 times per day, and some local businesses and government offices were present. Households were categorized as “rural” if they were located $> 5\text{km}$ from Bahir Dar city center, there was only 1 main paved road, there was

infrequent access to public transit to Bahir Dar city center, travel to the city center was primarily on foot or via donkey-drawn cart, and there were few or no local businesses or government offices in the area.

Household survey design

A semi-structured and standardized household survey with both closed and open ended questions was administered at 69 households. The survey was primarily comprised of closed ended categorical questions which were triangulated using open-ended questions and with observational data. The survey was originally written in English, translated to Amharic, and then back translated to English so we could evaluate the impact of translation on our intent and address discrepancies prior to piloting. The survey was designed to be 45 minutes in duration and to capture biometric, socio-economic, human and veterinary health data, as well as data on animal husbandry, manure management, and the division of household labor based on sex and age. Animal husbandry was defined as either extensive (all nutrition comes from the local environment with no provision of food and water), semi-intensive (most nutrition comes from the local environment, with frequent to occasional provision of food and water), and intensive (no nutrition comes from the local environment, all food and water is provided). Age intervals were established based on common trends in the published literature and cultural practices in the region and included: children < 5, children 5 – 13 years, and adults > 13 years.

The questions that centered on human health were designed to extract information on the history of diarrhea in the household. In order to minimize recall bias, specific timeframes were outlined in which participants would be more likely to accurately recall an event. Participants were asked about the household history of diarrhea in the last 2 weeks for all household members, and in the last 6 months for children < 5. Participants were also asked about the history of a clinic visit or hospitalization for diarrhea in the last 6 months. Since childhood illness, clinic visits, and hospitalizations are rare and memorable events a longer time frame was allotted for recall.

Piloting of the survey occurred over a period of 1 week in Bahir Dar, Ethiopia, and was conducted in Amharic. The survey was piloted and revised twice before data collection began.

Household survey administration and observational data collection

Two interviewers, one male and one female, were selected. Efforts were made to select individuals who did not openly project bias toward the study population, study objectives, and the role of women. Both interviewers were native Ethiopians, who spoke fluent English as well as fluent Amharic, and had at least 5 years' experience working in the local community. The male interviewer was a veterinarian affiliated with the ARGVS and The Donkey Sanctuary, and the female interviewer was a community health worker with expertise and graduate level education in gender studies. Both interviewers participated in piloting the survey and revising it.

Survey respondents were either the male or female head of household. Efforts were made to interview female respondents separately. On occasions when a female respondent was interviewed by the male interviewer, a female researcher or interviewer was always present. The survey was conducted entirely in Amharic and responses were recorded in English.

Information attained through the household survey was considered to be the main data source for this study. Observational data was used to supplement the household survey data. All observational data was collected by the primary researcher (first author, JAZ). Notes were taken during each interview both on the content of the interview, as well as on the physical features and characteristics of each household.

Informed consent was attained from all study participants. The Cornell University Institutional Review Board approved this study.

Statistical analysis

Data were analyzed using descriptive and inferential methods. Continuous data were determined to be non-Gaussian via the Shapiro–Wilk test. A Wilcoxon Rank Sum test was used to compare the initial age at which children are given responsibility for livestock to age and sex. Kruskal-Wallis was used to evaluate whether or not there were differences in the initial age at which children are given responsibility for livestock across setting and sex. Post-hoc analysis using the Wilcoxon Rank Sum test was carried out to determine differences between setting and sex.

Categorical explanatory variables were first explored for associations using the Chi-square or Fisher's exact test. Following the bivariate analysis, variables with a p-value ≤ 0.15 were retained for analysis via multiple variable logistic regression (Dohoo et al., 2003). Multiple variable logistic regression was carried out to identify relationships between the individual explanatory variables and 3 possible outcomes: household history of diarrhea during the previous 2 weeks, household history of diarrhea in children < 5 years of age in the previous 6 months, and household history of hospitalization or clinic visit for diarrhea in the previous 6 months. To arrive at the most parsimonious model, manual backward-stepwise regression was used to remove explanatory variables and their interactions when $p > 0.05$. Data were compiled and managed in MS Access (Microsoft Corporation, Redmond, WA) and analyzed using JMP 10 (SAS Institute Inc., 1989-2007).

Results

Study population

Sixty-nine households in urban ($n = 23$), peri-urban ($n = 24$), and rural ($n = 22$) settings were enrolled (Table 4.1). Fifty-three percent of the respondents were male and 47% were female. The education of respondents varied with 65% reporting no education, 13% reporting themselves to be literate and having completed some primary education, 13% having completed primary school, and 9% having some secondary education. All of the households had a tin roof, 52% had electricity, and 55% had a latrine. The primary water source for 23% of households was a river or lake. The remaining households used a protected water source such as a hand-pump or well (45%), or tap water (32%) as their main water source (Table 4.1). However, among households that reported using a protected water source, 17% also reported using a river or lake at the same time as their cattle when taking cattle to drink. On average, households owned 10 ± 7 livestock animals (median = 8, range 1- 42). Households with fewer than 8 animals were categorized as having a small livestock population, and those with 8 or more animals, were categorized as having a large livestock population. Presence of a small or large household livestock population did not

differ across setting ($p = 0.07$) though the data suggests that urban households tend to own fewer livestock. The median livestock population per household for cattle was 5 adults and 1 calf, but ranged from 0 to 13 and 0 to 3, respectively (Figure 4.1). All but 2 of the study households owned at least 1 adult cow. For sheep and lambs, the median population per household for both was 0, but ranged from 0 to 24 and 0 to 10, respectively. Rural and peri-urban households owned a median of 6 adult cattle, and urban households owned a median of 4.5. In all 3 settings, the median ownership of calves was 1 and for lambs was 0. The median ownership of adult sheep was 0 for rural and urban households and 1 for peri-urban households. The predominant husbandry system was semi-intensive (51%), and in 55% of households livestock were reported to sleep in the same room at night as the people (Table 4.1). When asked to provide an explanation of why livestock sleep in the house, almost all households cited tradition, stating “They just do.” In addition, the two most common reasons provided were: 1) to prevent theft of animals, and 2) to protect animals from inclement weather. Other explanations included: ease of manure collection, to prevent calves from nursing on dams, protection from foxes and hyenas, and lack of space to build a barn. In 62% of households manure was collected by hand. When asked about hand washing practices, 80% of households cleaned their hands with water only after handling manure, and 75% did not clean their hands at all after handling livestock (Table 4.1).

Explanatory Variable (n)		No. enrolled (%)	p - value		
			Outcome A	Outcome B	Outcome C
Location (69)	Urban	23 (33)	<0.0001^	<0.0001^	0.17
	Peri-urban	24 (35)			
	Rural	22 (32)			
Sex (68)	Male	36 (53)	0.9	0.9	0.1
	Female	32 (47)			
Education (68)	None	44 (65)	0.6	0.6	0.16
	Literate	9 (13)			
	Primary	9 (13)			
	Secondary	6 (9)			
Tin Roof (69)	No	0	-	-	-
	Yes	69 (100)			
Electricity (69)	No	33 (48)	0.1^	0.1^	0.3
	Yes	36 (52)			
Latrine (69)	No	31 (45)	0.5	0.5	0.15^
	Yes	38 (55)			
HH Water Source (69)	River/Lake	16 (23)	0.01^	0.01^	0.27
	Hand-pump or Well	31 (45)			
	Tap	22 (32)			
HH Livestock Population	< 8	18 (50)	0.5	0.5	0.9
	≥ 8	18 (50)			
Husbandry System (67)	Extensive	13 (19)	0.005^	0.1^	0.8
	Intensive	20 (30)			
	Semi-intensive	34 (51)			
Livestock sleeping location	Barn	16 (23)	0.4	0.16	0.3
	Inside the house- separate room	15 (22)			
	Inside the house- same room	38 (55)			
Child <5 yrs livestock (69)	No	54 (78)	0.7	0.7	0.5
	Yes	15 (22)			
Manure collection (69)	By hand	43 (62)	0.01^	0.01^	0.6
	Plant material	4 (6)			
	Both	22 (32)			
	Tool (shovel, etc)	0			
Hand washing (livestock) (67)	None	50 (75)	0.8	0.6	1.0
	Water only	15 (22)			
	Water & Soap	2 (3)			
Hand washing (manure) (69)	None	3 (4)	0.08	0.1^	0.5
	Water only	55 (80)			
	Water & Soap	11 (16)			
Diarrhea avoidance (69)	No	54 (78)	0.02^	0.4	0.04^
	Yes	15 (22)			
Hx of Malaria (69)	No	37 (54)	0.1^	0.7	0.6
	Yes	32 (46)			
Hx of Tuberculosis (69)	No	66 (96)	0.5	0.06^	0.4
	Yes	3 (4)			
Hx of HIV (69)	No	66 (96)	1.0	0.6	1.0
	Yes	3 (4)			

^ P value ≤ 0.15, predictor variable selected for inclusion in generation of final model

Table 4.1 Univariate analysis of risk factors from 69 households for 3 reported different outcomes: Household History of Diarrhea during the previous 2 weeks or not (Outcome A), Household history of diarrhea in children < 5 years of age in the previous 6 months or not (Outcome B), Household history of hospitalization or clinic visit for diarrhea in the previous 6 months or not and (Outcome C).

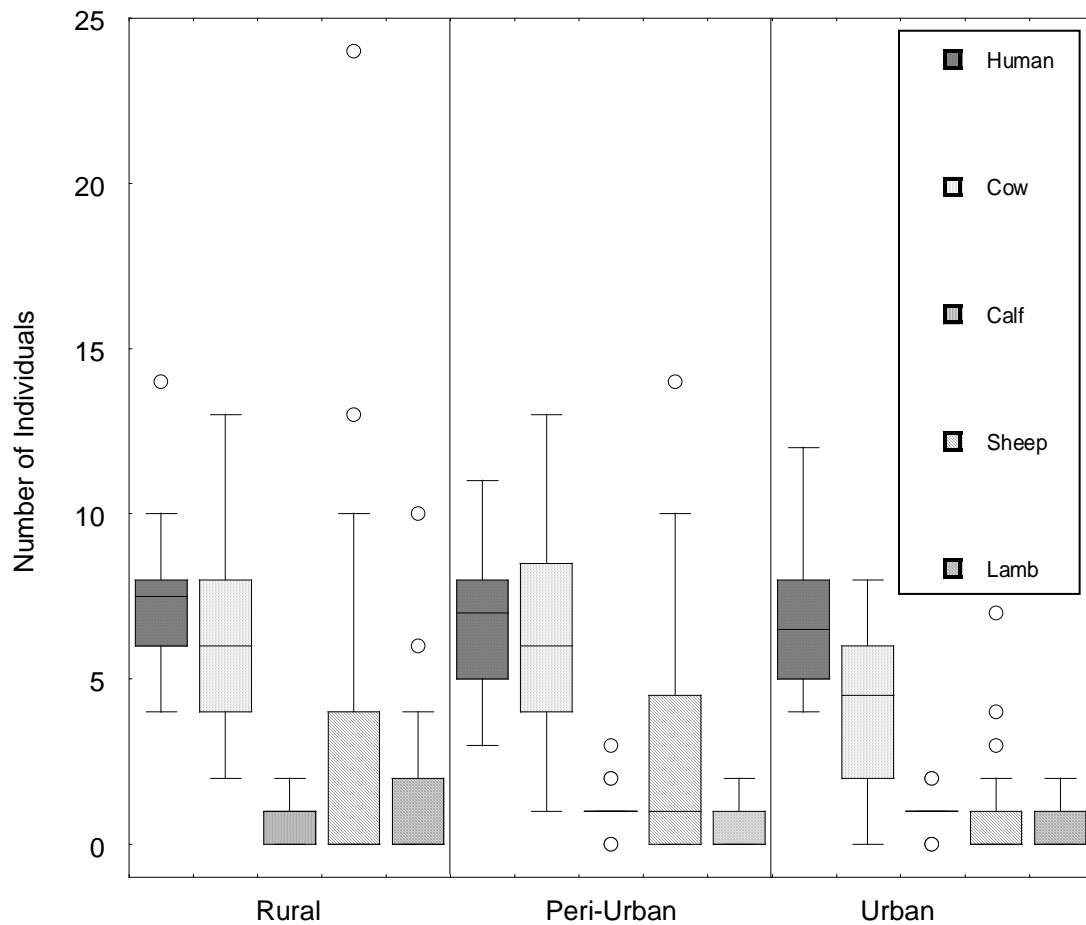


Figure 4.2 Household population of people and livestock by setting

Division of animal husbandry labor

With respect to the division of animal husbandry labor within the household, women ≥ 13 years of age are generally responsible for manure management, and men ≥ 13 years of age are generally responsible for grazing, taking animals to market, milking, care of sick animals, and slaughtering (Figure 4.2). Manure is managed and collected by women 74% of the time. This finding is consistent with field observations. When manure management was observed during household interviews, in each instance women (not men) were observed to be making compost, preparing manure for construction of floors and walls, or forming manure into patties for fuel. Male and female children up to 13 years of age were reported to collect manure in the fields and the house, and this was also observed during household interviews.

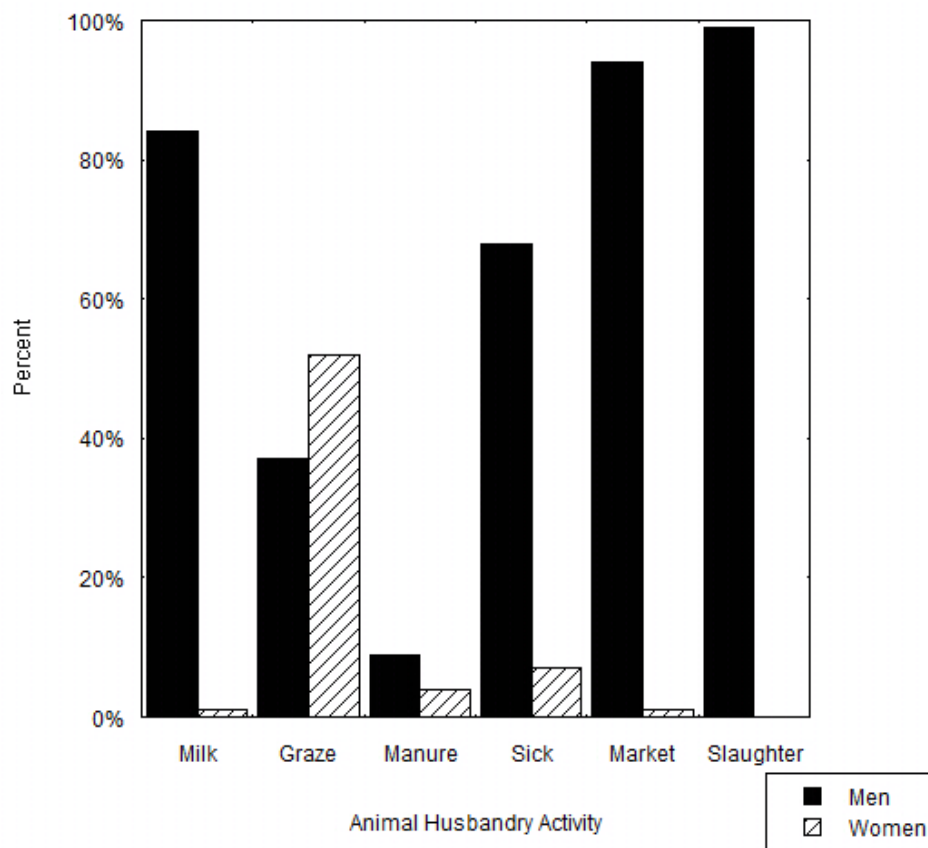


Figure 4.3 Division of animal husbandry labor by sex and activity

Children < 5 years of age

Children < 5 years of age also contribute to animal husbandry activities and are generally responsible for grazing, collecting manure, and care of sick animals. Field observations are consistent with these findings. In 22% of the study households, children <5 were assigned animal husbandry responsibilities and there was a difference between urban, peri-urban, and rural location of the household ($p = 0.0002$). Compared to children < 5 living in urban households, the odds of being engaged in animal husbandry were 30 times greater for children living in peri-urban households (95% CI 5.0-594; $p < 0.001$) and 8 times greater for children living in rural households (95% CI 1.1 -167; $p = 0.04$).

While children < 5 contribute to livestock husbandry, the median age at which children are initially given responsibility for livestock is slightly higher: 6 years for boys (range 2-14 years) and 7 years for girls (range 3-15 years). The initial age at which children are given responsibility for livestock

differed significantly across sex and the location of the household (urban, peri-urban, and rural) ($p < 0.001$) (Figure 4.3). Peri-urban male children caring for livestock were significantly younger than urban boys and urban girls ($p < 0.001$ for both), as well as rural boys ($p = 0.004$) and rural girls ($p = 0.05$). In addition, there was also a significant difference between rural male children caring for livestock and urban boys and urban girls ($p < 0.001$ for both).

For children who are ≥ 5 years of age the division of animal husbandry activities is similar to children < 5 , and is generally restricted to grazing, collecting manure, and care of sick animals. However, boys ≥ 5 are given relatively more responsibility for the grazing of livestock and this accounts for 52% of their contribution to animal husbandry within the household.

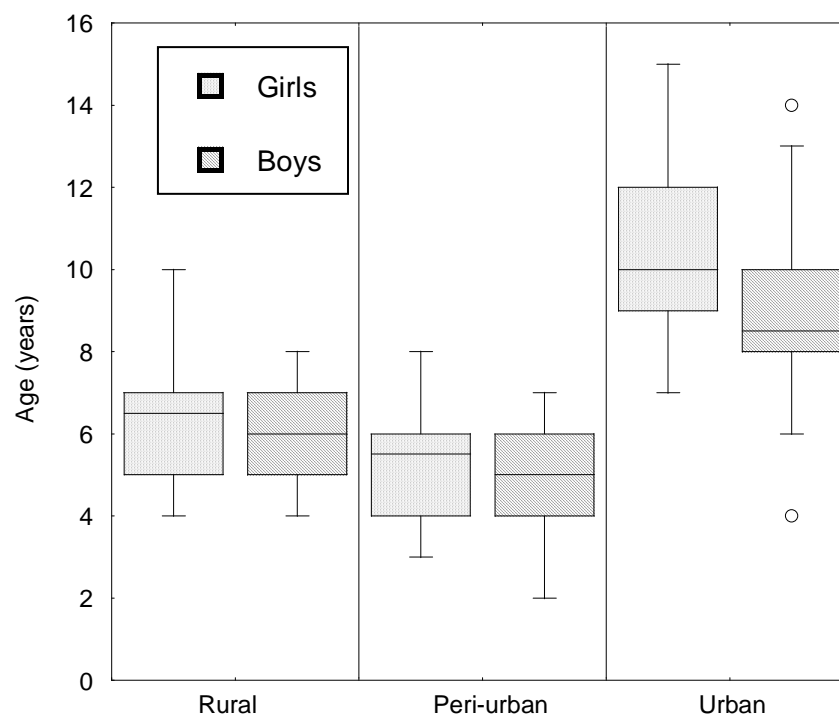


Figure 4.4 Initial age at which children are given responsibility for livestock by setting and sex

Description of diarrhea history

Among the 69 households, 19% reported that at least 1 member of the household had diarrhea in the previous 2 weeks and 58% reported that at least 1 head of livestock had diarrhea in the previous 2 weeks. When asked about the household history of diarrhea in the previous 6 months, 16% of households

reported that at least 1 child < 5 had diarrhea in the previous 6 months, and 40% reported that at least 1 individual in the household had been treated for diarrhea at a clinic or hospital.

There was not an association between history of diarrhea in the previous 2 weeks in livestock and household history of diarrhea in the previous 2 weeks ($p = 0.18$) or history of diarrhea in children < 5 in the previous 6 months ($p = 0.7$). There was a significant association between husbandry system and reported history of diarrhea in the previous 2 weeks. The odds of reporting diarrhea in the previous 2 weeks for 2.4×10^7 times greater for households that engage in intensive management, as compared to extensive management ($p = 0.01$, 95% CI 2 – Not Calculated), and there 3.4×10^7 times greater when compared to semi-intensive management ($p = 0.0001$, 95% CI 4 - NC). History of diarrhea in children < 5 in the previous 6 months did differ significantly across location ($p < 0.001$). In rural households, the odds of children < 5 having diarrhea in the previous 6 months was 5.7×10^7 times greater compared to children living in urban households (95% CI 6.7 – NC; $p < 0.001$) (NC) and 16 times greater compared to children living in peri-urban households (95% CI 2.6 – 305, $p = 0.001$).

Twenty-two percent of households reported that they actively engaged in behaviors that they believed reduced their risk of getting diarrhea (Table 4.1). When posed with an open-ended question and asked to describe the diarrhea avoidance activities in which the household engages, the most common activities reported were observing good personal hygiene, not consuming spoiled food, not consuming uncooked meat (a common cultural practice), and cleanliness in food preparation, but also included: traditional medicine, using a latrine, and consuming clean water. None of the respondents specifically stated “hand-washing” as a diarrhea avoidance activity, though this may be perceived to be part of personal hygiene and cleanliness in food preparation. There was an association between engaging in diarrhea avoidance behaviors and location ($p = 0.03$). Compared to urban households, rural households were 10.5 times more likely to engage in diarrhea avoidance behaviors (95% CI 1.7 – 205; $p = 0.009$) and peri-urban households were 7.4 times more likely (95% CI 1.1 – 147; $p = 0.04$). Compared to households that said they did not engage in diarrhea avoidance behaviors, in households that did say they engaged in

diarrhea avoidance behaviors the odds of reporting a history of diarrhea in the previous 2 weeks were 4.5 times greater (95% CI 1.2 – 17; $p = 0.03$). However, in multivariable analysis, where the outcome variable of interest was household history of diarrhea in the previous 2 weeks, and the explanatory variables were household setting (urban, peri-urban, or rural), engaging in diarrhea avoidance behaviors, and their interaction, only setting was significant ($p = 0.01$) (Table 4.2).

Variable	Coefficient (SE)		Odds Ratio (95% CI)	p - value
Setting				0.0003
Urban	Referent		Referent	Referent
Peri-urban	-4.9 (637)		1.2×10^7 (1.1 - NC)	0.04
Rural	-6.5 (637)		5.7×10^7 (7.7 - NC)	< 0.0001
Intercept	6.8 (637)		-	1.0

Table 4.2 Outcome A: Multivariable logistic regression model explaining the household history of diarrhea during the previous 2 weeks

Description of the association between explanatory variables and diarrhea history

Univariate logistic regression was carried out with 16 individual explanatory variables and each of 3 possible outcomes. The explanatory variables are listed in Table 1 and the possible outcomes were: household history of diarrhea during the previous 2 weeks or not, household history of diarrhea in children < 5 years of age in the previous 6 months or not, and household history of hospitalization or clinic visit for diarrhea in the previous 6 months or not. For all 3 outcomes, the explanatory variables education, tin roof, household livestock population, livestock sleeping location, children < 5 participating in livestock-keeping, hand washing after handling livestock, and HIV+ status all had p -values > 0.15 and were not retained for generation of the final models (Table 4.1).

Household history of diarrhea in the previous 2 weeks

Household history of diarrhea during the previous 2 weeks or not, had 7 explanatory variables retained (setting, electricity, household water source, husbandry system, method of manure collection, engaging in diarrhea avoidance behaviors, and household history of malaria in the previous 6 months).

From those 7 variables and their possible interactions, only the variable location ($p = 0.0004$) was selected for inclusion in the final model (Table 4.2).

Household history of diarrhea in children < 5 years of age in the previous 6 months

Household history of diarrhea in children < 5 years of age in the previous 6 months or not, 6 variables were retained (location, electricity, household water source, husbandry system, method of manure collection, and engaging in diarrhea avoidance behaviors). From those 6 variables and their possible interactions, only the variable location ($p < 0.001$) was retained for inclusion in the final model (Table 4.3).

Variable	Coefficient (SE)	Odds Ratio (95% CI)	p - value
Setting			<0.0001
Urban	Referent	Referent	Referent
Peri-urban	-4.1 (637)	3.7×10^6 (NC)	0.2
Rural	-6.9 (637)	5.7×10^7 (NC)	<0.0001
Intercept	7.2	-	1.0

Table 4.3 Outcome B: Multivariable logistic regression model explaining the history of diarrhea in children < 5 years of age in the previous 6 months

Household history of hospitalization or clinic visit for diarrhea in the previous 6 months

Household history of hospitalization or clinic visit for diarrhea in the previous 6 months or not, had 4 explanatory variables retained (sex, latrine, hand-washing after handling manure, and household history of a tuberculosis diagnosis in the previous 6 months). Hand-washing after handling manure and household history of a tuberculosis diagnosis in the previous 6 months were excluded. Sex ($p = 0.07$), latrine ($p = 0.1$), and the interaction of sex and latrine ($p = 0.04$) were retained for inclusion in the final model (Table 4.4).

Variable	Coefficient (SE)		Odds Ratio (95% CI)	p - value
Sex	Male	Referent	Referent	0.07
	Female	0.5 (0.3)	2.7 (0.9 – 8.2)	0.06
Latrine	Yes	Referent	Referent	0.1
	No	0.4 (0.3)	2.25 (0.8 – 6.7)	0.1
Sex & Latrine Interaction		0.6 (0.3)	-	0.04
Intercept		-0.4 (0.3)	-	0.01

Table 4.4 Outcome C: Multivariable logistic regression model explaining the history of hospitalization of clinic visit for diarrhea in the previous 6 mos (adults, young adults, and children < 5years old)

Final models describing the association between explanatory variables and diarrhea history

Household history of diarrhea during the previous 2 weeks and the history of diarrhea in children < 5 years of age in the previous 6 months were both best explained by the household setting (urban, peri-urban, or rural). Compared to urban households, the odds of household history of diarrhea in the previous 2 weeks were 1.2×10^7 (1.1 – NC; $p = 0.04$) times greater for peri-urban households and 5.7×10^7 (7.7 – NC; $p < 0.0001$) times greater for rural households (Table 4.2). Also compared to urban households, the odds of history of diarrhea in children < 5 years of age in the previous 6 months were 5.7×10^7 (95% CI NC; $p < 0.001$) times greater for rural households (Table 4.3).

Household history of hospitalization or clinic visit for diarrhea in the previous 6 months was best explained by the variables sex, latrine, and their interaction. In the final model, the odds of hospitalization or clinic visit were 2.7 times greater for female members of households that did not have a latrine (95% CI 0.9 – 8.2; $p = 0.06$), and the interaction between sex and latrine was significant ($p = 0.04$) (Table 4.4). In the stratified analysis, females with access to a latrine were 7 times more likely to have no reported history of hospital or clinic visit for diarrhea in the previous 6 mos (95% CI 1.7 – 34; $p = 0.01$) (Table 4.5).

Sex		Coefficient (SE)	Odds Ratio (95% CI)	p - value
Female				
	Latrine	-1.0 (0.4)	7 (1.7 – 34)	0.01
Male				
	Latrine	0.2 (0.4)	0.7 (0.15 – 2.7)	0.6

Table 4.5 The effect of having a latrine on the history of hospitalization or clinic visit for diarrhea in the previous 6 mos for females and males

Discussion

Animal husbandry and manure management activities among Ethiopian agro-pastoralists vary with age and sex, and are influenced in part by the needs of the family, local culture and custom, as well as traditional gender roles. Among our study participants, women and girls managed the manure, men were responsible for slaughter and market activities, and young boys contributed to herding. All of these activities constitute contact with livestock, which is a reported risk factor associated with transmission of zoonotic enteric pathogens (Grados et al., 1988; Fayer, 2004; Rao Ajjampur et al., 2007; Kotloff et al., 2013). Our study found that with the exception of manure management, all reported livestock husbandry activities are carried out by men, including the care of sick livestock. This is in keeping with gender roles in which men care for livestock and women care for poultry, though poultry were not included in this study.

In children < 5, gender roles are not as evident, and both boys and girls are frequently observed collecting manure for the household and assisting in herding livestock. The age at which children are initially given responsibility for the care of livestock varied across setting, with children living in urban areas assuming responsibility at an older age. Nevertheless, we found that children of all ages are engaged in livestock activities, and that this began as early as 2 years of age.

Given the wide range of activities and the variation across age and sex, it is plausible that not all types of contact with livestock pose an equivalent risk of disease transmission and not all

populations of people are at equal risk of becoming exposed. Herding cattle and collecting manure may not be equally likely to result in disease transmission; similarly, a young boy may be less likely than his mother to become exposed to a zoonotic diarrheal pathogen. Future research focused on zoonotic diarrheal disease in any human population should include an assessment of age and gender roles with respect to specific livestock husbandry activities.

The level and extent of contact with livestock not only varies depending on the type activity being carried out, but can also vary with the type of management system being used. Extensive, semi-intensive, and intensive production systems require varying levels of inputs and interaction with livestock, as such they can be treated as 3 different levels of livestock contact, ranging from minimal to frequent. Our study found an association between the type of production system in place and reported diarrhea history in our study households, however, in the regression analysis, husbandry system was not determined to be the best predictor of reported diarrhea history. We also did not find an association between history of diarrhea in livestock in the previous 2 weeks and reported history of diarrhea in people during the previous 2 weeks. It could be that there was insufficient contact with sick livestock for disease transmission to occur, or that our sample size was too small to detect it. However, our findings demonstrate that all members of the household, regardless of their age or sex, have contact with livestock each day, and that there are multiple opportunities for exposure and disease transmission. Thus, it may be that repeated exposure to these pathogens has protective effect, and that living in close proximity with livestock may be beneficial in this respect.

Instead of contact with livestock, our study suggests that household location is a more important determinant of diarrheal disease history. The odds of children < 5 having diarrhea in the previous 6 mos was 5.7×10^7 times greater in rural and peri-urban households. The confidence intervals on these estimates were wide, and are likely best explained by a small sample size, but even at the lower ends of these intervals, the impact is quite meaningful. Given

that the household population of livestock and livestock husbandry system did not differ significantly across household setting, it is unlikely that contact with livestock explains this observation. However, this difference could be explained by the fact that 100% of urban households have access to tap water, and that none of the peri-urban and rural households do. It could be argued that since children in urban households are given responsibility for livestock at an older age, they contribute less to animal husbandry and have less contact with livestock. However, these children live in close proximity to livestock. In 41% of urban households, the livestock sleep inside with the family.

The regression analysis in this study further supports the finding that household setting better explains reported diarrheal history than does contact with livestock. When controlling for multiple other variables, such as livestock sleeping location, animal husbandry system, and household livestock population; household setting emerges as the most important determinant of reported diarrheal history. This is likely associated with access to an improved water source in urban settings, but could also be associated with improved access to many other resources in urban settings.

While a stratified random sample would have been ideal, it was not possible to identify a sampling frame within the region. Therefore, we elected to use a purposive convenient sample while being cognizant of potential biases. Care should be taken when generalizing these study findings to other populations of African agro-pastoralists outside of Ethiopia. Given these limitations, this study does identify important trends that should be considered in the design of future research, regardless of the human population being studied. In general, other studies have reported that contact with livestock is associated with zoonotic disease transmission, however, those studies are not completely transferable to the context of agro-pastoral farming in Ethiopia,

and do not account for the impact of traditional gender roles and age. Not all types of livestock contact pose an equivalent risk of disease transmission, and some populations of people may be at greater risk of exposure depending upon their sex. In addition, living in close proximity to livestock may have a protective effect in some individuals with respect to transmission of zoonotic enteropathogens, and development of improved immunity. While contact with livestock is a risk factor for zoonotic disease transmission, our findings suggest that household setting is a more important factor when diarrheal outcomes are of primary concern (Grados et al., 1988; Adah et al., 2002; Fayer, 2004; Rao Ajjampur et al., 2007; Coles et al., 2009; Febriani et al., 2009). Therefore, future research focused on zoonotic diarrheal disease should include an assessment of age and gender roles in specific livestock husbandry activities, as well as the degree and extent of urbanization at the study site and in the surrounding area.

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Appendix

4.1 Household Survey Tool

I. Location Data

Date: _____ (DD/MMM/YY)	Farm ID: _____
City/Town: _____	Kebele: _____
GPS Lat: _____	GPS Long: _____
Setting (select one): Urban <input type="checkbox"/> Peri-Urban <input type="checkbox"/> Rural <input type="checkbox"/>	

II. Demographic Data for Informant

Age (years): _____		Male <input type="checkbox"/> Female <input type="checkbox"/>
Single <input type="checkbox"/> Married <input type="checkbox"/> Widowed <input type="checkbox"/> Divorced <input type="checkbox"/>		Education Level: _____
Tin roof: Yes <input type="checkbox"/> No <input type="checkbox"/>	Electricity: Yes <input type="checkbox"/> No <input type="checkbox"/>	Latrine: Yes <input type="checkbox"/> No <input type="checkbox"/>

III. Household Data

1. How many people live here? _____
2. Please list the number of people living here according to their age and gender:
 - Men (14 years and older): _____
 - Young boys (6-13 yrs): _____
 - Women (14 years and older): _____
 - Young girls (6-13 yrs): _____
 - Children (5yrs and younger): _____
3. How many people participating in house or farm activities: Male: _____ Female: _____

4. How many animals do you have of each type:

Cow: _____ Young cow (less than 3 months old): _____

Sheep: _____ Young sheep (less than 3 months old): _____

Goat: _____ Young goat (less than 3 months old): _____

Donkey: _____ Young donkey (less than 3 months old): _____

5. Where do you get water for your family and for your livestock? (*select all that apply*)

	Family (cooking, cleaning, drinking)	Livestock
Community well		
Community hand pump		
River, lake, or stream		
Collect rain water		
Other (please list)		

If YES for River/Lake/Stream→

- How often do you use this river/lake/stream (select one):

Once a month ☐ Once a week ☐ Once a day ☐ More than once a day ☐

- What activities do you do at the river/lake/stream? (select all that apply)

Bathe ☐ Drink water ☐ Clean Clothes ☐ Collect water for the house ☐ Other ☐

- How often do you use the river/lake/stream at the same time your livestock are drinking (select one):

Not often ☐ Somewhat often ☐ Often ☐ Very often ☐ Extremely often ☐

IV. Animal Husbandry

6. What best describes the type of animal husbandry practiced here:

Intensive ☐

Extensive ☐

Semi-intensive ☐

7. In your family, who is primarily responsible for taking care of the:

	Men: ≥14yrs	Boys: 6-13yrs	Women: ≥14yrs	Girls: 6-13yrs	Children: ≤5yrs	Other: list the person
Milking						
Grazing						
Removing manure from the house						
Collecting manure outside the house and in the fields						
Care of sick animals						
Feeding sick animals						
Taking animals to market						
Slaughter of animals						

8. In your family, do children that are 5 years old and younger have household or farm responsibilities? Yes ☐ No ☐

➔ If yes, what are the 3 most important chores they complete?

1. _____

2. _____

3. _____

9. In your family, do children that are 5 years old and younger take care of livestock?

Yes ☐ No ☐

➔ If YES

Which animals do they care for? (*select all that apply*)

Cow: _____ Young cow (less than 3 months old): _____

Sheep: _____ Young sheep (less than 3 months old): _____

Goat: _____ Young goat (less than 3 months old): _____

Donkey: _____ Young donkey (less than 3 months old): _____

10. At what age are children in your house given responsibility to care for livestock?

- Boys _____ years Girls _____ years

11. Do you (household) collect animal manure for household or farm use? Yes ☐ No ☐

→ If YES

- How do you use the manure you collect:

1. Fuel or burning _____
2. Home construction _____
3. Compost/Fertilizer _____
4. Sell it _____
5. Other _____

- How do you collect manure? (*select all that apply*)

	Inside of the house	Outside the house or in the fields
By hand		
Using plants or leaves		
Using a tool (shovel)		
Other (list)		

12. If an animal has diarrhea, what do you do with the manure? (*select all that apply*)

Collect it ☐ Cover it with ash/dirt ☐ Wash it away with water ☐ Leave it or do nothing ☐ Other ☐

13. If you collect diarrhea manure, how do you collect it? (*select all that apply*)

By hand ☐ Using a plant/leaves ☐ Using a tool (shovel, etc) ☐ Other ☐

14. Do use the diarrhea manure the same way as normal manure? Yes ☐ No ☐

If NO→ What do you do with it: 1. _____ 2. _____ 3. _____

15. Do your livestock sleep in the house? Yes ☐ No ☐

If YES→

- Do they sleep in the same room with your family? Yes ☐ No ☐
- List the 3 most important reasons why your livestock sleep in the house?

1. _____
2. _____
3. _____

16. When livestock sleep in the house, does this impact the health of the people in the house?

Yes ☐ No ☐ I don't know ☐

If YES→

- Is this effect on people's health: Good ☐ Bad ☐ I don't know ☐
- List any reasons given: _____

17. When did your livestock most recently have diarrhea? (select one)

1. This week _____, which animal _____
2. Last week _____, which animal _____
3. Last month _____, which animal _____
4. 6 mos ago _____

18. In your house, which type of livestock have diarrhea the most frequently compared to other livestock you own? (select one)

Cow: _____ Young cow (less than 3 months old): _____
Sheep: _____ Young sheep (less than 3 months old): _____
Goat: _____ Young goat (less than 3 months old): _____
Donkey: _____ Young donkey (less than 3 months old): _____
No difference: _____ I'm not sure: _____

19. Do you keep your livestock in a barn? Yes ☐ No ☐

If YES→

- Who is primarily responsible for cleaning the barn in your family? (select one)

1. Men (14 years and older): _____
2. Young boys (6-13 yrs): _____
3. Women (14 years and older): _____
4. Young girls (6-13 yrs): _____
5. Children (5yrs and younger): _____
6. None of the above, we hire someone to clean: _____

- How frequently is manure removed from the barn?

Once a day ☐ Once a week ☐ Once a month ☐ Other ☐

- What do you do with the manure you remove from the barn?

Fuel or burning ☐ Home construction ☐ Compost or fertilizer ☐ Sell it ☐ Other ☐

- How do you remove manure from the barn?

By hand ☐ Using a plant/leaves ☐ Using a tool (shovel, etc) ☐ Other ☐

- If you use a tool, do you use this tool for other types of work at your house?

Yes ☐ No ☐

If YES→ List the types of work:

1. _____
2. _____
3. _____

V. Human Health

20. After you handle your livestock do you clean your hands? Yes ☐ No ☐

21. After you remove or collect manure do you clean your hands? Yes ☐ No ☐

a. If yes for 19 or 20, how do you clean your hands?

	After touching livestock	After collecting/removing manure
Brush or wipe my hands on my clothes, your body, a towel, or a plant		
Run my hands under water		
Wash my hands with soap and water		
Other (list)		

22. In your opinion, is having diarrhea a big problem for your family (select one)?

Not a problem ☐ Slight problem ☐ Problem ☐ Big problem ☐ Huge problem ☐

23. When did someone in your family most recently have diarrhea? (select one)

This week ☐ Last week ☐ Last month ☐ 6 mos ago ☐ More than 1yr ☐

24. In your past experience, how often do children under 5 yrs in your house have diarrhea (select one)?

Not often ☐ Somewhat often ☐ Often ☐ Very often ☐ Extremely often ☐

25. In your family, who has diarrhea most often out of everyone?

1. Men (14 years and older): _____
2. Young boys (6-13 yrs): _____
3. Women (14 years and older): _____
4. Young girls (6-13 yrs): _____
5. Children (5yrs and younger): _____
6. There is no difference, everyone is equally effected: _____

26. In your experience, what are 2 most common causes of diarrhea for you and your family?

1. _____
2. _____
3. I don't know _____

27. To your knowledge, have you or anyone in your family ever gotten diarrhea from your

livestock in the last year? Yes ☐ No ☐ I don't know ☐

If YES→ * Which animals do you suspect made any of you sick? (list all)

1. _____
2. _____
3. _____

28. Is there anything that you do to avoid getting diarrhea? Yes ☐ No ☐

If YES→ * Please list what you do to avoid getting diarrhea:

1. _____

2. _____

3. _____

29. During the last 6 months, have you or any member of your family been treated for diarrhea at:

Clinic ☐

CHW ☐

Traditional Healer ☐

Hospital ☐

None ☐

If YES→

- List gender and age for each person

○ _____

○ _____

30. Has anyone in your household been hospitalized for an illness other than diarrhea in the

last 6 months? Yes ☐ No ☐

If YES→

- Please list the illnesses:

1. _____

2. _____

31. In your opinion, how much do you think having diarrhea would impact the ability to complete chores and work (select one)?

No effect ☐

Some effect ☐

Has an effect ☐

Big effect ☐

Huge effect ☐

32. Which chores and work are not completed or are partially completed if a family member has diarrhea? (list them)

1. _____

2. _____

3. _____

33. Does anyone in your household take medication every day? Yes ☐ No ☐

If YES→

- How many pills do they take? _____
- How long have they been taking this/these medication(s)?

1 month ☐ 3 months ☐ 1 yr of less ☐ 5 years or less ☐

34. Where do you get your medicine?

Clinic ☐ Pharmacy ☐ Traditional Healer ☐ NGO or Government ☐ Other ☐

35. Do you pay for your medicine? Yes ☐ No ☐

36. Has anyone in your family ever been diagnosed with TB? Yes ☐ No ☐

If YES→

- How long ago? _____
- Were they treated with medication? Yes ☐ No ☐
- Is anyone currently being treated for TB in your family? Yes ☐ No ☐
 - List the person and age: _____

37. Have you or anyone in your family ever been tested for HIV/AIDS?

Yes ☐ No ☐ I don't know ☐

38. To your knowledge, is anyone in your family infected with HIV/AIDS?

Yes ☐ No ☐ I don't know ☐

a. If yes, please list the age and gender of each person that is infected:

1.Age_____ Gender_____

2.Age_____ Gender_____

3.Age_____ Gender_____

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

Cryptosporidiosis impacts the health and wellbeing of people and animals alike. Infection in immune-suppressed individuals can be life-threatening, and in resource-poor settings, infection in calves not only places people at risk, it can also threaten their livelihoods. This research explored the epidemiology of *C. parvum* infection in dairy calves, and demonstrated that a disproportionately small number of oocysts are needed to cause infection and clinical illness, which is in keeping with similar studies of *C. parvum* and *C. hominis* in human populations. Given this knowledge, and the understanding that many of the world's poor are dependent upon their livestock and live in close proximity with them, disease control poses a significant challenge. Elimination of *Cryptosporidium* from the environment is not feasible; therefore, efforts must be directed at reducing the risk of exposure to the parasite, and developing practical interventions that are contextually and culturally appropriate.

Conclusions

The first objective of this research was to describe the probability of *Cryptosporidium parvum* fecal oocyst shedding at different magnitudes of oral exposure, the pattern of fecal shedding over time, and factors affecting fecal shedding in dairy calves. It was determined that calves that received lower doses of oocysts began shedding later, stopped shedding sooner, and shed fewer log-total oocysts per gram of feces dry weight. However, calves that received higher doses exhibited more variability in the range of oocysts shed per gram of feces dry weight. Regardless of dose magnitude, all calves exhibited the same pattern of fecal shedding over time, i.e., shedding gradually inclined, reached a peak, and then declined. In addition, this research demonstrated that calves shed oocysts in their feces in quantities that far exceed the number of oocysts to which they are initially exposed.

This initial research enabled us to describe the basic epidemiology of *C. parvum* infection in calves, and also highlighted areas for future study. In our first study, calves were not challenged with a dose of oocysts under 1,000. Given our findings in that study that the dynamics of infection and fecal shedding are different at lower doses in calves, and knowing that the ID₅₀ for *C. parvum* infection in people is 132 oocysts, we decided to challenge calves at even lower doses and determined the ID₅₀ for *C. parvum* infection in calves. In conducting a dose-response study, we similarly found an inverse relationship between dose magnitude and the onset of fecal oocyst shedding. While there was not a positive relationship between log-dose and the log-total oocysts counted per gram of feces dry weight, there was a positive relationship between log-dose and diarrhea. However, when controlling for other factors, log-dose ceased to be a significant predictor of diarrhea. Instead, onset and cessation of fecal oocyst shedding were found to be the best predictors.

Having gathered this information, regression analysis was carried out to determine the ID₅₀ for *C. parvum* infection in calves. Three different values were calculated, based upon 3 different definitions: 1) fecal oocyst shedding alone, 2) diarrhea alone, and 3) fecal oocyst shedding with diarrhea. The respective ID₅₀ for each definition was 5.8 oocysts, 9.7 oocysts, and 16.6 oocysts. This brings to light some formidable challenges in the control of cryptosporidiosis. First, the number of oocysts shed by an infected calf largely exceeds the number of oocysts needed to induce that shedding. Secondly, a calf can shed oocysts without developing diarrhea, thus, a clinically normal calf could still be infectious and contaminating the surrounding environment. Therefore, it is impossible to eliminate *C. parvum* from the environment. And, in environments where *C. parvum* is present, it is impossible to prevent calves from becoming

exposed and infected. Therefore, the best means of disease control is at the calf level, through the provision of clean dry housing and adequate nutrition.

The third and final objective of this research was to translate the epidemiologic knowledge of cryptosporidiosis garnered on the farm and in the laboratory, into an applied field setting with Agro-pastoral farmers in northern Ethiopia. *Cryptosporidium* is one of many zoonotic enteric pathogens, and while there are some key differences in the epidemiology of cryptosporidiosis as compared to other infectious agents, likewise, there are many similarities. Transmission of *Cryptosporidium* is by fecal-oral contact, and this is also true for pathogens like *Salmonella*, *E. coli*, and *Campylobacter*. In developing countries, where people live in close proximity with their livestock, and are dependent upon them for their livelihood, the degree and frequency of contact with their livestock is different than in many developed nations. Contact with livestock is a risk factor for transmission of zoonotic enteric pathogens, but this assumes that collecting manure and milking a cow are equivalent events in terms of disease transmission risk.

Therefore, the final portion of this research focused on the differences in animal husbandry and manure management across age and sex in Ethiopian agro-pastoralists with respect to reported history of diarrhea. The study found that there were significant differences in the division of animal husbandry labor, with women responsible for 75% of the manure management. Men, on the other hand, were more likely to assume responsibility for tasks like milking, slaughter, and market activities. In 22% of households, children < 5 were given responsibility for livestock. When this was looked at across setting, it was found that the odds of being engaged in animal husbandry were 30 times greater for children living in peri-urban

households and 8 times greater for children living in rural households (as compared to children < 5 in urban households).

Although 19% of households reported a history of diarrhea in the previous 2 weeks, was not an association between 2 week history of diarrhea in people and 2 week history of diarrhea livestock. Likewise, there was not a significant association between 2 week history of diarrhea and the type of husbandry system used. Instead, history of diarrhea in the previous 2 weeks was significantly associated with household setting. Compared to urban households, the odds of household history of diarrhea in the previous 2 weeks were 1.2×10^7 times greater for peri-urban households and 5.7×10^7 times greater for rural households. In rural households, the odds of children < 5 having diarrhea in the previous 6 months was 5.7×10^7 times greater compared to children living in urban households and 16 times greater compared to children living in peri-urban households.

These findings generally indicate poorer conditions and health outcomes related to diarrhea for individuals living in rural and peri-urban settings. While zoonotic disease transmission cannot be ruled out entirely, this data suggests that other factors may be more influential in the transmission of diarrheal illness, specifically, access to protected water sources. All urban households in our study population had access to tap water, which is likely to impact correct observation of WASH activities (water, sanitation, and hygiene). Furthermore, urban households generally have improved access to other resources, and this could have a protective effect with respect to diarrheal disease transmission.

Lastly, we anticipated that a recent history of diarrhea in livestock would be associated with a recent history of diarrhea in people. We did not find this to be the case, and this might be

due to a relatively small sample size. However, in 55% of the study households, livestock sleep in the same house with people, and defecate inside the house as well. There is ample opportunity for disease transmission, and yet these study findings don't indicate that transmission is occurring. This could indicate a protective effect of living in close proximity to livestock, and increased immunity to common zoonotic enteric pathogens.

Future directions

Currently, there are no consistently effective and commercially available vaccines or chemotherapeutics for prevention or treatment of cryptosporidiosis. In cattle, there have been recent advances in the development of a colostral antibody vaccine, and for people who are immune-competent, treatment with nitazoxanide remains an option. However, this is not sufficient. As we consider the struggles faced in developing vaccinations against other Apicomplexan parasites, like malaria, and as we wait for advances in those fields, the need for prevention measures becomes of paramount importance.

Future research on *Cryptosporidium* should be directed at exploiting the fact that it is abundant, ubiquitous, and relatively easy to identify with rudimentary laboratory equipment. *Cryptosporidium* is a sentinel for fecal contamination and ingestion of fecal material. Thus, by identifying sources of *Cryptosporidium* infection in people and calves, it may also be possible to identify other points of transmission for other enteric pathogens. In taking this type of approach, it then becomes possible to identify points of intervention. Interventions should be simple and attainable within the local environment. Interventions that require large external inputs, specialized training to execute, are dependent upon stable governance and infrastructure, and which create additional work for women should immediately be rejected.

Furthermore, additional research should be directed at the division of animal husbandry labor within households. This research suggests that women are primarily responsible for the management of manure, and that young boys are disproportionately responsible for the care of livestock and this may be exaggerated in peri-urban households. Thus, these two populations of individuals may be at greater risk for different types of exposures. If this is not accounted for in future study designs, it may be possible to over or under estimate the likelihood of disease transmission in these populations.

Final remarks

I realize that the human-animal bond means different things to different people. In developed countries, our understanding of this bond is often limited to our relationship with our companion animals. But, in developing countries, this bond centers around the livestock that provide sustenance to families and secure their livelihoods. In the developing world livestock not only plough fields and fill milk cups, they educate children, they expand social networks, they pay for medicines, and they lift families out of poverty.

I firmly believe that through the promotion of livestock health and wellbeing, it is possible to promote human health and wellbeing. It is my sincere hope that in some small way this research benefits the livestock and the people who depend upon them.